DEVELOPMENT OF MUTATIONS USEFUL FOR ATTENUATING DENGUE VIRUSES AND CHIMERIC DENGUE VIRUSES

Related Applications

[0001] This application is a continuation and claims the benefit of priority of International Application No. PCT/US02/16308 filed May 22, 2002, designating the United States of America and published in English as WO 02/095075 on November 28, 2002, which claims the benefit of priority of U.S. Provisional Application No. 60/293,049 filed May 22, 2001, both of which are hereby expressly incorporated by reference in their entireties.

Field of the Invention

[0002] A menu of mutations was developed that is useful in fine-tuning the attenuation and growth characteristics of dengue virus vaccines.

Background of the Invention

[0003] Dengue virus is a positive-sense RNA virus belonging to the Flavivirus genus of the family Flaviviridae. Dengue virus is widely distributed throughout the tropical and semitropical regions of the world and is transmitted to humans by mosquito vectors. Dengue virus is a leading cause of hospitalization and death in children in at least eight tropical Asian countries (WHO, 1997. *Dengue haemorrhagic fever: diagnosis, treatment prevention and control* - 2nd ed. Geneva: WHO). There are four serotypes of dengue virus (DEN-1, DEN-2, DEN-3, and DEN-4) which annually cause an estimated 50 - 100 million cases of dengue fever and 500,000 cases of the more severe form of dengue virus infection, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (Gubler, D.J. & Meltzer, M. 1999 *Adv Virus Res* **53**:35-70). DHF/DSS is seen predominately in children and adults experiencing a second dengue virus infection with a serotype different than that of their first dengue virus infection and in primary infection of infants who still have circulating dengue-specific maternal antibody (Burke, D.S. *et al.* 1988 *Am J Trop Med Hyg* **38**:172-80; Halstead, S.B. *et al.* 1969 *Am J Trop Med Hyg* **18**:997-1021; Thein, S. *et al.* 1997 *Am J Trop Med Hyg* **56**:566-72). A vaccine is needed to lessen the disease burden caused by dengue virus, but none is licensed. Because of the association of more severe disease with secondary dengue virus infection, a successful vaccine must induce immunity to all four serotypes. Immunity is

primarily mediated by neutralizing antibody directed against the envelope E glycoprotein, a virion structural protein. Infection with one serotype induces long-lived homotypic immunity and a short-lived heterotypic immunity (Sabin, A. 1955 *Amer J Trop Med Hyg* **4**:198-207). Therefore, the goal of immunization is to induce a long-lived neutralizing antibody response against DEN-1, DEN-2, DEN-3, and DEN-4, which can best be achieved economically using live attenuated virus vaccines. This is a reasonable goal since a live attenuated vaccine has already been developed for the related yellow fever virus, another mosquito-borne flavivirus present in tropical and semitropical regions of the world (Monath, T.P. & Heinz, F.X. 1996 in: Fields B.N. *et al.* eds. *Fields Virology* Philadelphia: Lippincott-Ravan Publishers, 961-1034).

[0004] Several live attenuated dengue vaccine candidates have been developed and evaluated in humans or non-human primates. The first live attenuated dengue vaccine candidates were host range mutants developed by serial passage of wild type dengue viruses in the brains of mice and selection of mutants attenuated for humans (Kimura, R. & Hotta, S. 1944 *Japanese J Bacteriology* **1**:96-99; Sabin, A.B. & Schlesinger, R.W. 1945 *Science* **101**:640; Wisseman, C.L. Jr. *et al.* 1963 *Am J Trop Med* **12**:620-623). Although these candidate vaccine viruses were immunogenic in humans, their poor growth in cell culture discouraged further development. Additional live attenuated DEN-1, DEN-2, DEN-3, and DEN-4 vaccine candidates have been developed by serial passage in tissue culture (Angsubhakorn, S. *et al.* 1994 *Southeast Asian J Trop Med Public Health* **25**:554-9; Bancroft, W.H. *et al.* 1981 *Infect Immun* **31**:698-703; Bhamarapravati, N. *et al.* 1987 *Bull World Health Organ* **65**:189-95; Eckels, K.H. *et al.* 1984 *Am J Trop Med Hyg* **33**:684-9; Hoke, C.H. Jr. *et al.* 1990 *Am J Trop Med Hyg* **43**:219-26; Kanesa-thasan, N. *et al.* 2001 *Vaccine* **19**:3179-88) or by chemical mutagenesis (McKee, K.T. Jr. *et al.* 1987 *Am J Trop Med Hyg* **36**:435-42). It has proven very difficult to achieve a satisfactory balance between attenuation and immunogenicity for each of the four serotypes of dengue virus using these approaches and to formulate a tetravalent vaccine that is safe and satisfactorily immunogenic against each of the four dengue viruses (Kanesa-thasan, N. *et al.* 2001 *Vaccine* **19**:3179-88; Bhamarapravati, N. & Sutee, Y. 2000 *Vaccine* **18** Suppl 2: 44-7).

[0005] Two major advances utilizing recombinant DNA technology have recently made it possible to develop additional promising live attenuated dengue virus vaccine candidates. First, methods have been developed to recover infectious dengue virus from cells transfected with RNA transcripts derived from a full-length cDNA clone of the dengue virus genome, thus making it possible to derive infectious viruses bearing attenuating mutations which have been introduced into the cDNA clone by site-directed mutagenesis (Lai, C.J. *et al.* 1991 *PNAS USA* **88**:5139-43). Second, it is possible to produce antigenic chimeric viruses in which the structural protein coding region of the full-length cDNA clone of dengue virus is replaced by that of a different dengue virus serotype or from a more divergent flavivirus (Bray, M. & Lai, C.J. 1991 *PNAS USA* **88**: 10342-6; Chen, W. *et al.* 1995 *J Virol* **69**:5186-90; Huang, C.Y. *et al.* 2000 *J Virol* **74**:3020-8; Pletnev, A.G. & Men, R. 1998 *PNAS USA* **95**:1746-51). These techniques have been used to construct intertypic chimeric dengue viruses which have been shown to be effective in protecting monkeys against homologous dengue virus challenge (Bray, M. *et al.* 1996 *J Virol* **70**:4162-6). Despite these advances, there is a need to develop attenuated antigenic dengue virus vaccines that specify a satisfactory balance between attenuation and immunogenicity for humans.

Summary of the Invention

[0006] The invention provides mutations that confer temperature sensitivity in Vero cells or human liver cells, host-cell restriction in mosquito or human liver cells, host-cell adaptation for improved replication in Vero cells, or attenuation in mice, which mutations are useful in fine tuning the attenuation and growth characteristics of dengue virus vaccines.

Brief Description of the Drawings

[0007] **Figure 1** shows growth of wt DEN4 2A and vaccine candidate, 2AΔ30, in Vero and HuH-7 cells. Vero (A) or HuH-7 (B) cells were infected with DEN4 2A or 2AΔ30 at a multiplicity of infection (MOI) of 10 or 0.01. Confluent cell monolayers in 25-mm tissue culture flasks were washed and overlaid with a 1.5 ml inoculum containing the indicated virus. After a two hour incubation at 37°C, cells were washed three times in PBS and 7 ml of culture media supplemented with 2% FBS was added. A 1 ml aliquot of tissue culture medium was removed, replaced with fresh medium, and designated the 0 hour time-

point. At the indicated time points post-infection, samples of tissue culture media were removed and frozen at -70°C. The level of viral replication was assayed by plaque titration in Vero cells. Briefly, serial ten-fold dilutions of cell culture media samples were inoculated onto confluent Vero cell monolayers in 24-well plates in duplicate and overlaid with Opti-MEM containing 0.8% methylcellulose. After five days, plaques were visualized by immunoperoxidase staining as described in Example 1.

[0008] **Figure 2** shows generation of temperature-sensitive (*ts*) DEN4 viruses by 5-fluorouracil (5-FU) chemical mutagenesis. The wild-type DEN4 2A virus was derived from a cDNA clone of DEN4 strain 814669 (Dominica, 1981). Vero cells were infected with DEN4 2A and overlaid with culture media containing 1 mM 5-fluorouracil (5-FU) which resulted in a reduction of approximately 100-fold in viral replication when compared to untreated controls. Viral progeny from the 1 mM 5-FU-treated cultures were subjected to a single round of terminal dilutions generating 1,248 biologically cloned viruses which were screened for *ts* phenotypes by assessing virus replication at 35°C and 39°C in Vero and HuH-7 cells. Virus clones which demonstrated a 100-fold or greater reduction in titer at 39°C were terminally diluted an additional two times and amplified in Vero cells. Temperature-sensitive phenotypes of the 3x biologically cloned viruses were confirmed by evaluating efficiency of plaque formation (EOP) in the indicated cells as described in Example 1.

[0009] **Figure 3** shows plaque size phenotypes of representative 5-FU mutant DEN4 viruses. Serial ten-fold dilutions of wild-type DEN4 2A-13 (A), 5-FU mutant viruses #569 and #1189 (B), and 5-FU mutant viruses #1083 and #311 (C) were inoculated onto confluent Vero and HuH-7 cell monolayers in 24-well plates. After incubation at 35°C for two hours, monolayers were overlaid with 0.8% methylcellulose culture media. Following incubation at 35°C for five days, plaques were visualized by immunoperoxidase staining. Viruses which had a plaque size that was $\leq$1 mm (approximately $\leq$50% the size of wt DEN4 2A-13) at the permissive temperature of 35°C were designated as having the small-plaque (*sp*) phenotype. Mutant viruses #569 and #1189 (B) were *sp* in both Vero and HuH-7 cells, and #311 and #1083 (C) were *sp* in only HuH-7 cells.

[0010] **Figure 4** shows generation of recombinant DEN4 viruses. (A), The p4 cDNA clone is represented which was constructed from the 2A cDNA clone (derived from

DEN4 814669) by site-directed mutagenesis. Restriction enzyme sites were introduced or removed to facilitate subsequent cloning of DEN4 recombinants bearing introduced attenuating mutations. Restriction enzyme sites are shown and define fragments of the genome that were sub-cloned into modified pUC-119 vectors for site-directed mutagenesis to introduce mutations identified in the 5-FU mutant viruses. (B), An outline of the methods used to generate rDEN4 viruses is also represented and described in Example 1.

[0011] **Figure 5** shows amino acid sequence of the rDEN4 NS5 gene (SEQ ID NO: 1). Eighty underlined amino acid pairs were mutagenized to alanine pairs; 32 pairs in boldface represent mutant viruses that could be recovered in either Vero or C6/36 cells; pairs in normal type represent mutant viruses that could not be recovered in either Vero or C6/36 cells. Boxed regions indicate putative functional domains, including an S-adenosylmethionine utilizing methyltransferase domain (SAM), an importin-β binding domain adjacent to a nuclear localization sequence (importin-β - binding + NLS) and an RNA-dependent RNA polymerase domain (Polymerase).

[0012] **Figure 6** shows plaque size of mutant 5-1A1 in C6/36 cells. Note that 5-1A1 has a small plaque phenotype in C6/36 cells relative to that of the wild type virus.

[0013] **Figure 7** shows growth of wild type rDEN4 and 5-1A1 in C6/36 cells. Cells were inoculated in triplicate with each virus at an MOI of 0.01, and the amount of virus present in the supernatants that were harvested on the indicated days was determined by plaque enumeration in Vero cells. The titers are expressed as $\log_{10}$PFU/ml ± standard error.

[0014] **Figure 8** shows nucleotide alignment of the 3' UTR of mosquito-borne and tick-borne flaviviruses. cDNA sequences are shown 5' to 3' and represent a portion of the UTR corresponding to DEN4 nucleotides 10417 to 10649 (3' genome end). Nucleotide numbering represents the position in the alignment. Regions deleted or swapped are indicated using the nucleotide numbering of DEN4. GenBank accession numbers for mosquito-borne viruses: DEN4 (SEQ ID NO: 2): AF326825, DEN1 (SEQ ID NO: 3): U88535, DEN2 (SEQ ID NO: 4): AF038403, DEN3 (SEQ ID NO: 5): M93130, West Nile virus (WN) (SEQ ID NO: 6): M12294, Japanese encephalitis virus (JE) (SEQ ID NO: 7): AF315119, Yellow fever virus (YF) (SEQ ID NO: 8): U17067; GenBank accession numbers for tick-borne viruses: Powassan virus (POW) (SEQ ID NO: 9): L06436, Louping Ill virus

(LI) (SEQ ID NO: 10): Y07863, Tick-borne encephalitis virus (TBE) (SEQ ID NO: 11): U27495, and Langat virus (LGT) (SEQ ID NO: 12): AF253419.

[0015] **Figure 9** shows genetic map of plasmid p4. Dengue cDNA is shown as bold line, with the C-prM-E region exchanged during construction of chimeric dengue virus cDNAs indicated.

[0016] **Figure 10** shows plaque size phenotypes of rDEN4 viruses encoding Vero adaptation mutations. Serial three-fold dilutions of the indicated viruses were inoculated onto confluent Vero and C6/36 cell monolayers in 6-well plates. After incubation at 37°C (Vero) or 32°C (C6/36) for two hours, monolayers were overlaid with 0.8% methylcellulose culture media. Following incubation for five days, plaques were visualized by immunoperoxidase staining. Values below each well are the average plaque size in mm ± standard error. For each of the virus-infected wells, 36 plaques were measured on the digital image of the 6-well plate on Adobe Photoshop at 300% view.

[0017] **Figure 11** shows growth curve in Vero cells of rDEN4 viruses encoding single Vero adaptation mutations. Vero cells were infected with the indicated viruses at an MOI of 0.01. Confluent cell monolayers in 25-cm^2 tissue culture flasks were washed and overlaid with a 1.5 ml inoculum containing the indicated virus. After a two hour incubation at 37°C, cells were washed three times in PBS and 5 ml of culture medium supplemented with 2% FBS was added. A 1 ml aliquot of tissue culture medium was removed, replaced with fresh medium, and designated the 0 hour time-point. At the indicated time points post-infection, samples of tissue culture medium were removed, clarified, and frozen at -70°C. The level of virus replication was assayed by plaque titration in Vero cells. Briefly, serial ten-fold dilutions of cell culture media samples were inoculated onto confluent Vero cell monolayers in 24-well plates in duplicate and overlaid with Opti-MEM containing 0.8% methylcellulose. After five days, plaques were visualized by immunoperoxidase staining as described in Example 1. Limit of detection (L.O.D.) is ≥ 0.7 $\log_{10}$PFU/ml.

[0018] **Figure 12** shows growth curve in Vero cells of rDEN4 viruses encoding combined Vero cell adaptation mutations. Vero cells were infected with the indicated viruses at an MOI of 0.01. Confluent cell monolayers in 25-cm^2 tissue culture flasks were washed and overlaid with a 1.5 ml inoculum containing the indicated virus. After a two hour

incubation at 37°C, cells were washed three times in PBS and 5 ml of culture medium supplemented with 2% FBS was added. A 1 ml aliquot of tissue culture medium was removed, replaced with fresh medium, and designated the 0 hour time-point. At the indicated time points post-infection, samples of tissue culture medium were removed, clarified, and frozen at -70°C. The level of virus replication was assayed by plaque titration in Vero cells. Limit of detection (L.O.D.) is ≥ 0.7 $\log_{10}$PFU/ml.

Brief Description of the Tables

[0019] **Table 1.** Susceptibility of mice to intracerebral DEN4 infection is age-dependent.

[0020] **Table 2.** Temperature-sensitive (*ts*) and mouse brain attenuation (*att*) phenotypes of 5-FU mutant DEN4 viruses.

[0021] **Table 3.** Nucleotide and amino acid differences of the 5-FU mutant viruses which are *ts* in both Vero and HuH-7 cells.

[0022] **Table 4.** Nucleotide and amino acid differences of the 5-FU mutant viruses which are *ts* in only HuH-7 cells.

[0023] **Table 5.** Mutations which are represented in multiple 5-FU mutant DEN4 viruses.

[0024] **Table 6.** Addition of *ts* mutation 4995 to rDEN4Δ30 confers a *ts* phenotype and further attenuates its replication in suckling mouse brain.

[0025] **Table 7.** Temperature-sensitive (*ts*) and mouse brain attenuation (*att*) phenotypes of 5-FU DEN4 mutant viruses which exhibit a small plaque (*sp*) phenotype.

[0026] **Table 8.** Viruses with both *ts* and *sp* phenotypes are more restricted in replication in mouse brain than those with only a *ts* phenotype.

[0027] **Table 9.** Nucleotide and amino acid differences of the 5-FU mutant DEN4 viruses which produce small plaques in both Vero and HuH-7 cells.

[0028] **Table 10.** Nucleotide and amino acid differences of the 5-FU mutant DEN4 viruses which produce small plaques in only HuH-7 cells.

[0029] **Table 11.** Putative Vero cell adaptation mutations derived from the full set of 5-FU mutant viruses.

[0030] **Table 12.** Mutagenic oligonucleotides used to generate recombinant DEN4 viruses containing single 5-FU mutations.

[0031] **Table 13.** *sp*, *ts* and mouse attenuation phenotypes of rDEN4 mutant viruses encoding single mutations identified in six *sp* 5-FU mutant viruses.

[0032] **Table 14.** Phenotypes of rDEN4 mutant viruses encoding single mutations identified in 10 5-FU mutant viruses that are *ts* in both Vero and HuH-7 cells.

[0033] **Table 15.** *sp*, *ts* and mouse attenuation phenotypes of rDEN4 mutant viruses encoding single mutations identified in 3 HuH-7 cell-specific *ts* 5-FU mutant viruses.

[0034] **Table 16.** Temperature-sensitive (*ts*) and mouse brain attenuation (*att*) phenotypes of additional rDEN4 viruses encoding single 5-FU mutations.

[0035] **Table 17.** Growth of wt DEN-4 2A-13 in SCID mice transplanted with HuH-7 cells.

[0036] **Table 18.** Combination of *ts* mutations, NS3 4995 and NS5 7849, in rDEN4 results in an additive *ts* phenotype.

[0037] **Table 19.** The 5-FU mutations are compatible with the Δ30 mutation for replication in the brain of suckling mice.

[0038] **Table 20.** Temperature-sensitive and mouse brain attenuation phenotypes of viruses bearing charge-cluster-to-alanine mutations in the NS5 gene of DEN4.

[0039] **Table 21.** SCID-HuH-7 attenuation phenotypes of viruses bearing charge-cluster-to-alanine mutations in the NS5 gene of DEN4.

[0040] **Table 22.** Combination of paired charge-cluster-to-alanine mutations into double-pair mutant viruses.

[0041] **Table 23.** Temperature-sensitive and mouse brain attenuation phenotypes of double charge-cluster-to-alanine mutants of the NS5 gene of rDEN4.

[0042] **Table 24.** SCID-HuH-7 attenuation phenotypes of double charge-cluster-to-alanine mutants of the NS5 gene of rDEN4.

[0043] **Table 25.** Phenotypes (temperature sensitivity, plaque size and replication in mouse brain and SCID-HuH-7 mice) of wt DEN4 and viruses containing the Δ30 and 7129 mutations.

[0044] **Table 26.** The 5-fluorouracil 5-1A1 small plaque mutant demonstrates a restriction of midgut infection following oral infection of *Aedes aegypti* mosquitoes.

[0045] **Table 27.** The 5-fluorouracil 5-1A1 small plaque mutant demonstrates a restriction of infection following intrathoracic inoculation of *Toxorhynchites splendens* mosquitoes.

[0046] **Table 28.** Mutagenesis primers for the deletion or swap of sequences in DEN4 showing conserved differences from tick-borne flaviviruses.

[0047] **Table 29.** Virus titer and plaque size of 3' UTR mutant viruses in Vero and C6/36 cells.

[0048] **Table 30.** Infectivity of wt DEN4 and 3' UTR mutants for *Toxorhynchites splendens* via intrathoracic inoculation.

[0049] **Table 31.** Infectivity of 3' UTR swap mutant viruses for *Aedes aegypti* fed on an infectious bloodmeal.

[0050] **Table 32.** Putative Vero cell adaptation mutations derived from the set of 5-FU mutant viruses and other DEN4 viruses passaged in Vero cells.

[0051] **Table 33.** Sequence analysis of rDEN2/4Δ30 clone 27(p4)-2-2A2.

[0052] **Table 34.** Sequence analysis of rDEN2/4Δ30 clone 27(p3)-2-1A1.

[0053] **Table 35.** Recombinant virus rDEN2/4Δ30 bearing Vero adaptation mutations can be recovery and titered on Vero cells.

[0054] **Table 36.** Putative Vero cell adaptation mutations of dengue type 4 virus and the corresponding wildtype amino acid residue in other dengue viruses.

[0055] **Table 37.** Mutations known to attenuate dengue type 4 virus and the corresponding wildtype amino acid residue in other dengue virus.

Brief Description of the Appendices

[0056] **Appendix 1.** Sequence of recombinant dengue type 4 virus strain 2A (amino acid sequence SEQ ID NO: 13 and nucleotide sequence SEQ ID NO: 14).

[0057] **Appendix 2.** Sequence of recombinant dengue type 4 virus strain rDEN4 (amino acid sequence SEQ ID NO: 15 and nucleotide sequence SEQ ID NO: 16).

[0058] **Appendix 3.** Sequence of recombinant dengue type 2 chimeric virus strain rDEN2/4Δ30 (amino acid sequence SEQ ID NO: 17 and nucleotide sequence SEQ ID NO: 18).

[0059] **Appendix 4.** Alignment of dengue virus polyproteins. DEN4 (SEQ ID NO: 19); DEN1-WP (SEQ ID NO: 20); DEN2-NGC (SEQ ID NO: 21); DEN3-H87 (SEQ ID NO: 22).

Detailed Description of the Preferred Embodiment

[0060] To assemble a collection of useful mutations for incorporation in recombinant live dengue virus vaccines, site-directed and random mutagenesis techniques were used to introduce mutations into the dengue virus genome. The resulting mutant viruses were screened for several valuable phenotypes, including temperature sensitivity in Vero cells or human liver cells, host cell restriction in mosquito cells or human liver cells, host-cell adaptation for improved replication in Vero cells, and attenuation in mice. The genetic basis for each observed phenotype was determined by direct sequence analysis of the virus genome. Mutations identified through these sequencing efforts have been further evaluated by their re-introduction, singly, or in combination, into recombinant dengue virus and characterization of the resulting phenotypes. In this manner, a menu of mutations was developed that is useful in fine-tuning the attenuation and growth characteristics of dengue virus vaccines.

Example 1

Chemical Mutagenesis of Dengue Virus Type 4 Yields Temperature-Sensitive and Attenuated Mutant Viruses

[0061] A recombinant live attenuated dengue virus type 4 (DEN4) vaccine candidate, 2AΔ30, was found previously to be generally well-tolerated in humans, but a rash and an elevation of liver enzymes in the serum occurred in some vaccinees. 2AΔ30, a non-temperature-sensitive (*ts*) virus, contains a 30 nucleotide deletion in the 3' untranslated region (UTR) of the viral genome. In the present study, chemical mutagenesis of DEN4 has been utilized to generate attenuating mutations which may be useful to further attenuate the incompletely attenuated 2AΔ30 candidate vaccine. Wild-type DEN4 2A virus was grown in Vero cells in the presence of 5-fluorouracil, and, from a panel of 1,248 clones that were

isolated in Vero cells, twenty *ts* mutant viruses were identified which were *ts* in both Vero and HuH-7 cells (n = 13) or in HuH-7 cells only (n = 7). Each of the twenty *ts* mutations possessed an attenuation (*att*) phenotype as indicated by restricted replication in the brains of seven day old mice. The complete nucleotide sequence of the 20 *ts* mutant viruses identified nucleotide substitutions in structural and non-structural genes as well as in the 5' and 3' UTR with more than one change occurring, in general, per mutant virus. A *ts* mutation in the NS3 protein (nucleotide position 4,995) was introduced into a recombinant DEN4 virus possessing the Δ30 deletion creating the rDEN4Δ30-4995 recombinant virus which was found to be *ts* and to be more attenuated than rDEN4Δ30 in the brains of mice. A menu of attenuating mutations is being assembled that should be useful in generating satisfactorily attenuated recombinant dengue vaccine viruses and in increasing our understanding of the pathogenesis of dengue virus.

[0062] The mosquito-borne dengue (DEN) viruses (serotypes 1 to 4) are members of the *Flavivirus* genus and contain a single-stranded positive-sense RNA genome of approximately 10,600 nucleotides (nt) (Monath, T.P. & Heinz, F.X. 1996 in: *Fields Virology* B.N. Fields, *et al.* Eds. pp. 961-1034 Lippincott-Ravan Publishers, Philadelphia). The genome organization of DEN viruses is 5'-UTR-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-UTR-3' (UTR – untranslated region, C – capsid, PrM – pre-membrane, E – envelope, NS – non-structural) (Chang, G.-J. 1997 in: *Dengue and dengue hemorrhagic fever* D.J. Gubler & G. Kuno, eds. pp. 175-198 CAB International, New York; Rice, C.M. 1996 in: *Fields Virology* B.N. Fields *et al.* Eds. pp. 931-959 Lippincott-Raven Publishers, Philadelphia). A single viral polypeptide is co-translationally processed by viral and cellular proteases generating three structural proteins (C, M, and E) and seven NS proteins. The disease burden associated with DEN virus infection has increased over the past several decades in tropical and semitropical countries. Annually, there are an estimated 50-100 million cases of dengue fever (DF) and 500,000 cases of the more severe and potentially lethal dengue hemorrhagic fever / dengue shock syndrome (DHF/DSS) (Gubler, D.J. & Meltzer, M. 1999 *Adv Virus Res* **53**:35-70).

[0063] The site of viral replication in DEN virus-infected humans and the pathogenesis of DF and DHF/DSS are still incompletely understood (Innis, B.L. 1995 in:

Exotic viral infections J.S. Porterfield, ed. pp. 103-146 Chapman and Hall, London). In humans, DEN virus infects lymphocytes (Kurane, I. *et al.* 1990 *Arch Virol* **110**:91-101; Theofilopoulos, A.N. *et al.* 1976 *J Immunol* **117**:953-61), macrophages (Halstead, S.B. *et al.* 1977 *J Exp Med* **146**:218-29; Scott, R.M. *et al.* 1980 *J Infect Dis* **141**:1-6), dendritic cells (Libraty, D.H. *et al.* 2001 *J Virol* **75**:3501-8; Wu, S.J. *et al.* 2000 *Nat Med* **6**:816-20), and hepatocytes (Lin, Y.L. *et al.* 2000 *J Med Virol* **60**:425-31; Marianneau, P. *et al.* 1996 *J Gen Virol* **77**:2547-54). The liver is clearly involved in DEN virus infection of humans, as indicated by the occurrence of transient elevations in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in the majority of dengue virus-infected patients and by the presence of hepatomegaly in some patients (Kalayanarooj, S. *et al.* 1997 *J Infect Dis* **176**:313-21; Kuo, C.H. *et al.* 1992 *Am J Trop Med Hyg* **47**:265-70; Mohan, B. *et al.* 2000 *J Trop Pediatr* **46**:40-3; Wahid, S.F. *et al.* 2000 *Southeast Asian J Trop Med Public Health* **31**:259-63). DEN virus antigen-positive hepatocytes are seen surrounding areas of necrosis in the liver of fatal cases (Couvelard, A. *et al.* 1999 *Hum Pathol* **30**:1106-10; Huerre, M.R. *et al.* 2001 *Virchows Arch* **438**:107-15), and dengue virus sequences were identified in such cases using RT-PCR (Rosen, L. *et al.* 1999 *Am J Trop Med Hyg* **61**:720-4). Of potential importance to the etiology of severe dengue virus infection, three studies have demonstrated that the mean levels of serum ALT/AST were significantly increased in patients with DHF/DSS versus those with DF (Kalayanarooj, S. *et al.* 1997 *J Infect Dis* **176**:313-21; Mohan, B. *et al.* 2000 *J Trop Pediatr* **46**:40-3; Wahid, S.F. *et al.* 2000 *Southeast Asian J Trop Med Public Health* **31**:259-63).

[0064] A vaccine for DEN viruses is not presently licensed. Since previous infection with one dengue virus serotype can increase the risk for DHF/DSS following infection with a different serotype (Burke, D.S. *et al.* 1988 *Am J Trop Med Hyg* **38**:172-80; Halstead, S.B. *et al.* 1969 *Am J Trop Med Hyg* **18**:997-1021; Thein, S. *et al.* 1997 *Am J Trop Med Hyg* **56**:566-72), it is clear that a dengue virus vaccine will need to protect against each of the four dengue virus serotypes, namely DEN1, DEN2, DEN3, and DEN4. Several strategies are currently being actively pursued in the development of a live attenuated tetravalent DEN virus vaccine (Bancroft, W.H. *et al.* 1984 *J Infect Dis* **149**:1005-10; Bhamarapravati, N. & Sutee, Y. 2000 *Vaccine* **18**:44-7; Guirakhoo, F. *et al.* 2000 *J Virol*

74:5477-85; Huang, C.Y. *et al.* 2000 *J Virol* **74**:3020-8). Recently, we demonstrated that a live attenuated DEN4 vaccine candidate, 2AΔ30, was attenuated and immunogenic in a group of 20 human volunteers (see Example 8). This recombinant DEN4 virus contains a 30 nt deletion in the 3' UTR which removes nucleotides 10,478-10,507 and was restricted in replication in rhesus monkeys. Levels of viremia in humans were low or undetectable, and virus recovered from the vaccinees retained the Δ30 mutation. An asymptomatic rash was reported in 50% of patients. The only laboratory abnormality observed was an asymptomatic, transient rise in the serum ALT level in 5 of 20 vaccinees. All vaccinees developed serum-neutralizing antibody against DEN4 virus (mean titer: 1:580). Importantly, 2AΔ30 was not transmitted to mosquitoes fed on vaccinees and has restricted growth properties in mosquitoes (Troyer, J.M. *et al.* 2001 *Am J Trop Med Hyg* **65**:414-9). The presence of a rash and of the elevated ALT levels suggests that the 2AΔ30 vaccine candidate is slightly under-attenuated in humans. Because of the overall set of desirable properties conferred by the Δ30 mutation, chimeric vaccine candidates are being constructed which contain the structural genes of dengue virus type 1, 2, and 3 and the DEN4 attenuated backbone bearing the genetically stable Δ30 mutation.

[0065] Although the initial findings indicate the utility of the 2AΔ30 vaccine candidate, many previous attempts to develop live attenuated dengue virus vaccines have yielded vaccine candidates that were either over- or under-attenuated in humans (Eckels, K.H. *et al.* 1984 *Am J Trop Med Hyg* **33**:684-9; Bhamarapravati, N. & Yoksan, S. 1997 in: *Dengue and dengue hemorrhagic fever* D.J. Gubler & G. Kuno eds. pp. 367-377 CAB International, New York; Innis, B.L. *et al.* 1988 *J Infect Dis* **158**:876-80; McKee, K.T., Jr. *et al.* 1987 *Am J Trop Med Hyg* **36**:435-42). Therefore, we developed a menu of point mutations which confer temperature-sensitive (*ts*) and attenuation (*att*) phenotypes upon DEN4. These mutations are envisioned as being useful to attenuate DEN4 viruses to different degrees and therefore as having purpose in fine-tuning the level of attenuation of vaccine candidates such as 2AΔ30. Addition of such mutations to 2AΔ30 or to other dengue virus vaccine candidates is envisioned as resulting in the generation of a vaccine candidate that exhibits a satisfactory balance between attenuation and immunogenicity for humans.

[0066] In the present example, chemical mutagenesis of DEN4 has been utilized to identify point mutations which confer the *ts* phenotype, since such viruses often are attenuated in humans. Additionally, because of the reported involvement of the liver in natural dengue infection and the elevated ALT levels in a subset of 2AΔ30 vaccinees, mutagenized DEN4 viruses were also evaluated for *ts* phenotype in HuH-7 liver cells derived from a human hepatoma. Here, we describe the identification of 20 DEN4 *ts* mutant viruses each of which replicates efficiently in Vero cells, the proposed substrate for vaccine manufacture, and each of which is attenuated in mice. Finally, the feasibility of modifying the attenuation phenotype of the 2AΔ30 vaccine candidate by introduction of a point mutation in NS3 is demonstrated.

[0067] **Cells and viruses.** WHO Vero cells (African green monkey kidney cells) were maintained in MEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Summit Biotechnologies, Fort Collins, CO), 2 mM L-glutamine (Life Technologies), and 0.05 mg/ml gentamicin (Life Technologies). HuH-7 cells (human hepatoma cells) (Nakabayashi, H. *et al.* 1982 *Cancer Res* **42**:3858-63) were maintained in D-MEM/F-12 (Life Technologies) supplemented with 10% FBS, 1 mM L-glutamine and 0.05 mg/ml gentamicin. C6/36 cells (*Aedes albopictus* mosquito cells) were maintained in complete MEM as described above supplemented with 2 mM non-essential amino acids (Life Technologies).

[0068] The wild type (wt) DEN4 2A virus was derived from a cDNA clone of DEN4 strain 814669 (Dominica, 1981) (Men, R. *et al.* 1996 *J Virol* **70**:3930-7). Sequence of the cDNA of DEN 4 2A virus is presented in Appendix 1. The full-length 2A cDNA clone has undergone several subsequent modifications to improve its ability to be genetically manipulated. As previously described, a translationally-silent *Xho*I restriction enzyme site was engineered near the end of the E region at nucleotide 2348 to create clone 2A-*Xho*I (Bray, M. & Lai, C.J. 1991 *PNAS USA* **88**:10342-6). The viral coding sequence of the 2A-*Xho*I cDNA clone was further modified using site-directed mutagenesis to create clone p4: a unique *Bbv*CI restriction site was introduced near the C-prM junction (nucleotides 447 - 452); an extra *Xba*I restriction site was ablated by mutation of nucleotide 7730; and a unique *Sac*II restriction site was created in the NS5 region (nucleotides 9318 - 9320). Each of these

engineered mutations is translationally silent and does not change the amino acid sequence of the viral polypeptide. Also, several mutations were made in the vector region of clone p4 to introduce or ablate additional restriction sites. The cDNA clone p4Δ30 was generated by introducing the Δ30 mutation into clone p4. This was accomplished by replacing the *Mlu*I - *Kpn*I fragment of p4 (nucleotides 10403 - 10654) with that derived from plasmid 2AΔ30 containing the 30 nucleotide deletion. The cDNA clones p4 and p4Δ30 were subsequently used to generate recombinant viruses rDEN4 (Appendix 2) and rDEN4Δ30, respectively. (The GenBank accession number for rDEN4 is AF326825 and the accession for rDEN4Δ30 is AF326827).

[0069] Chemical mutagenesis of DEN4. Confluent monolayers of Vero cells were infected with wt DEN4 2A at an multiplicity of infection (MOI) of 0.01 and incubated for 2 hours at 32°C. Infected cells were then overlaid with MEM supplemented with 2% FBS and 5-fluorouracil (5-FU) (Sigma, St. Louis, MO) at concentrations ranging from 10 mM to 10 nM. After incubation at 32°C for five days, cell culture medium was harvested, clarified by centrifugation, and frozen at -70°C. Clarified supernatants were then assayed for virus titer by plaque titration in Vero cells. Serial ten-fold dilutions of the clarified supernatant were prepared in Opti-MEM I (Life Technologies) and inoculated onto confluent Vero cell monolayers in 24-well plates. After incubation at 35°C for two hours, monolayers were overlaid with 0.8% methylcellulose (EM Science, Gibbstown, NJ) in Opti-MEM I supplemented with 2% FBS, gentamicin, and L-glutamine. Following incubation at 35°C for five days, plaques were visualized by immunoperoxidase staining. Vero cell monolayers were fixed in 80% methanol for 30 minutes and washed for 10 minutes with antibody buffer which consists of 3.5% (w/v) nonfat dry milk (Nestle, Solon, OH) in phosphate buffered saline (PBS). Cells were then incubated for one hour at 37°C with an anti-DEN4 rabbit polyclonal antibody preparation ($PRNT_{50}$ of >1:2000) diluted 1:1,000 in antibody buffer. After one wash with antibody buffer, cells were incubated for one hour with peroxidase-labeled goat-anti-rabbit IgG (KPL, Gaithersburg, MD) diluted 1:500 in antibody buffer. Monolayers were washed with PBS, allowed to dry briefly, overlaid with peroxidase substrate (KPL), and plaques were counted.

[0070] Virus yields in cultures treated with 1 mM 5-FU were reduced 100-fold compared to untreated cultures, and the virus present in the supernatant from the 1 mM 5-FU-treated culture was terminally diluted to derive clones for phenotypic characterization. Briefly, 96 well plates of Vero cells were inoculated with the 5-FU-treated virus at an MOI that yielded 10 or fewer virus-positive wells per plate. After a five-day incubation at 35°C, cell culture media from the 96 well plates were temporarily transferred to 96 well plates lacking cells, and the positive cultures were identified by immunoperoxidase staining of the infected-cell monolayers. Virus from each positive well was transferred to confluent Vero cell monolayers in 12 well plates for amplification. Cell culture medium was harvested from individual wells five or six days later, clarified by centrifugation, aliquoted to 96 deep-well polypropylene plates (Beckman, Fullerton, CA) and frozen at –70°C. A total of 1,248 virus clones were prepared from the 1 mM 5-FU-treated cultures. Two wt virus clones, 2A-1 and 2A-13, were generated in the same manner from the 5-FU untreated control cultures.

[0071] **Screening of clones for *ts* and *att* phenotypes.** The 1,248 virus clones were screened for *ts* phenotype by assessing virus replication at 35°C and 39°C in Vero and HuH-7 cells. Cell monolayers in 96 well plates were inoculated with serial ten-fold dilutions of virus in L-15 media (Quality Biologicals, Gaithersburg, MD) supplemented with 2% FBS, L-glutamine and gentamicin. Cells were incubated at the indicated temperatures for five days in temperature-controlled water baths, and presence of virus was determined by immunoperoxidase staining as described above. Virus clones which demonstrated a 100-fold or greater reduction in titer at 39°C were terminally diluted an additional two times and amplified in Vero cells. The efficiency of plaque formation (EOP) at permissive and restrictive temperatures of each triply biologically cloned virus suspension was determined as follows. Plaque titration in Vero and HuH-7 cells was performed as described above except virus-infected monolayers were overlaid with 0.8% methylcellulose in L-15 medium supplemented with 5% FBS, gentamicin, and L-glutamine. After incubation of replicate plates for five days at 35, 37, 38, or 39°C in temperature-controlled water baths, plaques were visualized by immunoperoxidase staining and counted.

[0072] The replication of DEN4 5-FU *ts* mutant viruses was evaluated in Swiss Webster suckling mice (Taconic Farms, Germantown, NY). Groups of six one-week-old

mice were inoculated intracranially with 10^4 PFU of virus diluted in 30 µl Opti-MEM I. Five days later, mice were sacrificed and brains were removed and individually homogenized in a 10% suspension of phosphate-buffered Hank's balanced salt solution containing 7.5% sucrose, 5 mM sodium glutamate, 0.05 mg/ml ciprofloxacin, 0.06 mg/ml clindamycin, and 0.0025 mg/ml amphotericin B. Clarified supernatants were frozen at -70°C and subsequently virus titer was determined by titration in Vero cells, and plaques were stained by the immunoperoxidase method described above.

[0073] Sequence analysis of viral genomes. The nucleotide sequence of the 5-FU-mutagenized DEN4 viruses was determined. Briefly, genomic viral RNA was isolated from virus clones with the QIAamp viral RNA mini kit (Qiagen, Valencia, CA) and reverse transcription was performed using the SuperScript First Strand Synthesis System for RT-PCR (Life Technologies) and random hexamer primers. Advantage cDNA polymerase (Clontech, Palo Alto, CA) was used to generate overlapping PCR fragments of approximately 2,000 nt which were purified by HighPure PCR Product Purification System (Roche Diagnostics, Indianapolis, IN). DEN-specific primers were used in BigDye terminator cycle sequencing reactions (Applied Biosystems, Foster City, CA) and reactions were analyzed on a 3100 genetic analyzer (Applied Biosystems). Primers were designed to sequence both strands of the PCR product from which consensus sequences were assembled.

[0074] The nucleotide sequence of the 5' and 3' regions of the viral genome were determined as above after circularization of the RNA genome. The 5' cap nucleoside of the viral RNA was excised using tobacco acid pyrophosphatase (Epicentre Technologies, Madison, WI) and the genome was circularized by RNA ligase (Epicentre Technologies). A RT-PCR fragment was generated which overlapped the ligation junction (5' and 3' ends) and was sequenced as described above.

[0075] Generation of recombinant DEN4 viruses. The mutation at nt position 4,995 in NS3 was introduced into the p4 cDNA construct by site-directed mutagenesis (Kunkel, T.A. 1985 *PNAS USA* **82**:488-92). The *Stu*I – *Bst*BI (nt 3,619 – 5,072) fragment of p4 was sub-cloned into a modified pUC119 vector. The U > C mutation at nt position 4,995 was engineered by site-directed mutagenesis into the p4 fragment, cloned back into the p4 cDNA construct, and the presence of the mutation was confirmed by sequence analysis. The

Δ30 mutation was introduced into the 3' UTR of the p4-4995 cDNA clone by replacing the *Mlu*I – *Kpn*I fragment with that derived from the p4Δ30 cDNA clone, and the presence of the deletion was confirmed by sequence analysis. Full length RNA transcripts were prepared from the above cDNA clones by *in vitro* transcription. Briefly, transcription consisted of a 50 µl reaction mixture containing 1 µg linearized plasmid, 60 U SP6 polymerase (New England Biolabs (NEB), Beverly, MA), 1X RNA polymerase buffer (40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol), 0.5 mM m7G(5′)ppp(5′)G cap analog (NEB), 1 mM each nucleotide triphosphate, 1 U pyrophosphatase (NEB), and 80 U RNAse inhibitor (Roche, Indianapolis, IN). This reaction mixture was incubated at 40°C for 90 min and the resulting transcripts were purified using RNeasy mini kit (Qiagen, Valencia, CA).

[0076] For transfection of C6/36 cells, RNA transcripts were combined with DOTAP liposomal transfection reagent (Roche) in HEPES-buffered saline (pH 7.6) and added to cell monolayers in 6 well plates. After incubation at 32°C for 12-18 hours, the cell culture media were removed and replaced with MEM supplemented with 5% FBS, L-glutamine, gentamicin and non-essential amino acids. Cell monolayers were incubated for an additional 5 to 7 days and cell culture media were harvested, clarified by centrifugation, and assayed for the presence of virus by plaque titration in Vero cells. Recovered viruses were terminally diluted twice as described above, and virus suspensions for further analysis were prepared in Vero cells.

[0077] ***In vitro* (tissue culture) and *in vivo* replication of wt DEN4 and DEN4Δ30.** The level of replication of both wt DEN4 2A and the vaccine candidate, 2AΔ30, was evaluated in Vero (monkey kidney) and HuH-7 (human hepatoma) cells (Figure 1), the latter of which has recently been found to efficiently support the replication of DEN2 virus (Lin, Y.L. *et al.* 2000 *J Med Virol* **60**:425-31). The pattern of replication of wt DEN4 2A and 2AΔ30 was similar in both cell lines. Viral titers from cultures infected with 2AΔ30 at an MOI of 0.01 were slightly reduced compared to wt DEN4 2A at 72 hours, but at later time points their level of replication was equivalent. The efficient replication of both DEN4 viruses in each cell line indicated that these continuous lines of cells would be useful for characterization of the *ts* phenotype of the 1248 potential mutant viruses.

[0078] The level of replication of DEN4 virus administered intracerebrally to Swiss Webster mice was first determined to assess whether mice could be used to efficiently evaluate and quantitate the attenuation phenotype of a large set of mutant viruses. Since the susceptibility of mice to DEN infection is age dependent (Cole, G.A. & Wisseman, C.L.Jr. 1969 *Am J Epidemiol* **89**:669-80; Cole, G.A. *et al.* 1973 *J Comp Pathol* **83**:243-52), mice aged 7 to 21 days were infected with 2A-13 (a clone of DEN4 wild type virus - see below), rDEN4 or rDEN4Δ30, and after five days the brain of each mouse was removed, and the level of viral replication was quantitated by plaque assay (Table 1). The results indicated that the two wt DEN4 viruses and the rDEN4Δ30 vaccine candidate replicated to high titer (>6.0 $\log_{10}$PFU/g brain) in 7-day old mice and that the mean viral titers were similar among the three viruses. These results demonstrated the feasibility of using 7-day old mice to screen a large set of mutant viruses, and the high level of replication of wild type and vaccine candidate permits one to quantitate the magnitude of the restriction of replication specified by an attenuating mutation over a 10,000-fold range.

[0079] **Generation and *in vitro* characterization of DEN4 5-FU mutant viruses.** A panel of 1,248 DEN4 virus clones was generated from a 5-FU-mutagenized suspension of wt DEN4 2A as described above (Figure 2). Each clone was tested in Vero and HuH-7 cells for the *ts* phenotype at 39°C, and putative *ts* mutant viruses were subjected to two additional rounds of biological cloning by terminal dilution, and the *ts* phenotype of each further cloned virus population was examined in more detail by determining their efficiency of plating (EOP) at permissive temperature (35°C) and at various restrictive temperatures (Table 2). One virus (clone 2A-13) without a *ts* phenotype, which was passaged in an identical fashion as the *ts* mutant viruses, served as the virus to which each of the *ts* mutant viruses was directly compared for both the *ts* and *att* phenotypes.

[0080] Thirteen 5-FU mutant viruses were identified which have a *ts* phenotype in both Vero and HuH-7 cells, and seven mutant viruses were *ts* only in HuH-7 cells (Table 2). Mutant viruses which were *ts* in Vero cells but not in HuH-7 cells were not identified. Temperature-sensitivity was defined as a $\geq$2.5 or $\geq$3.5 $\log_{10}$PFU/ml reduction in virus titer in Vero or HuH-7 cells, respectively, at an indicated temperature when compared to the permissive temperature of 35°C. Wild type DEN4 2A was found to have approximately a

0.5 and 1.5 $\log_{10}$PFU/ml reduction in virus titer in Vero or HuH-7 cells at 39°C, respectively. The Δ30 deletion did not confer a *ts* phenotype in Vero or HuH-7 cells and exhibited only a slight reduction in virus titer (2.2 $\log_{10}$PFU/ml) at 39°C in HuH-7 cells, which was less than 10-fold greater than the reduction of wt DEN4 2A at that temperature. Several 5-FU mutant viruses had a greater than 10,000-fold reduction in virus titer at 39°C in both Vero and HuH-7 cells. A complete shut-off in viral replication at 39°C in HuH-7 cells was observed in five virus clones (#571, 605, 631, 967, and 992) which were not *ts* in Vero cells. Mutations that selectively restrict replication in HuH-7 liver cells may be particularly useful in controlling the replication of dengue virus vaccine candidates in the liver of vaccinees.

[0081] Replication of DEN4 5-FU mutant viruses in suckling mice. The level of replication of each of the 20 *ts* DEN4 mutant viruses in mouse brain was determined (Table 2). The titers obtained were compared to that of the two wt viruses, 2A-13 and rDEN4, which each replicated to a level of greater than 10^6 PFU/g of brain tissue, and to that of the 2AΔ30 mutant, which conferred only a limited 0.5 $\log_{10}$PFU/g reduction in mean virus titer compared to the wt controls. The observed reduction in the level of rDEN4Δ30 replication was consistent among 11 separate experiments. Interestingly, the rDEN4Δ30 virus, which was attenuated in both rhesus monkeys and humans (Example 8), was only slightly restricted in replication in mouse brain. Varying levels of restriction of replication were observed among the mutant viruses ranging from a 10-fold (#473) to over 6,000-fold (#686) reduction. Mutant viruses with *ts* phenotypes in both Vero and HuH-7 cells, as well as in HuH-7 cells alone, were found to have significant *att* phenotypes. Five of 13 5-FU mutant viruses with *ts* phenotypes in both Vero and HuH-7 cells and five of seven mutant viruses with *ts* phenotypes in HuH-7 cells alone had greater than a 100-fold reduction in virus replication. There appeared to be no direct correlation between the magnitude of the reduction in replication at restrictive temperature in tissue culture and the level of attenuation *in vivo*. The similar level of temperature sensitivity and replication of the rDEN4 wt and clone 2A-13 in mouse brain indicated that observed differences in replication between the *ts* mutant viruses and clone 2A-13 was not simply a function of passage in Vero cells, but reflects the sequence differences between these viruses.

[0082] **Sequence analysis of DEN4 5-FU mutant viruses.** To determine the genetic basis of the observed *ts* and *att* phenotypes, the complete nucleotide sequence of each *ts* mutant and of clone 2A-13 was determined and summarized in Table 3 (*ts* in Vero and HuH-7 cells) and Table 4 (*ts* in only HuH-7 cells).

[0083] The only type of mutation identified in the 20 mutant viruses sequenced was a nucleotide substitution (no deletions or insertions occurred), and these were present in each of the coding regions except C and NS4A. Three mutant viruses (#239, 489, and 773) contained only a single missense point mutation in NS3 at nt position 4,995 resulting in a Ser to Pro amino acid (a.a.) change at a.a. position 1,632. For #773, this was the sole mutation present (Table 3). The non-coding mutations in coding regions are not considered to be significant. The 17 additional mutant viruses had multiple mutations (two to five) in a coding region or in an UTR which could potentially confer the observed *ts* or *att* phenotypes. Five of the 17 mutant viruses with multiple mutations (#473, 718, 759, 816, and 938) also encoded the point mutation at nt position 4,995. The presence of the 4,995 mutation was found in only DEN4 mutant viruses with *ts* phenotypes in both Vero and HuH-7 cells.

[0084] The sequence analysis indicated that 10 mutant viruses which were *ts* in Vero and HuH-7 cells and three mutant viruses which were *ts* in only HuH-7 cells contained mutations in only the 5' and 3' UTR and/or in a nonstructural protein. These mutations are especially suitable for inclusion in chimeric dengue virus vaccine candidates in which the structural genes derive from a DEN1, DEN2, or DEN3 serotype and the remaining coding and non-coding regions come from an attenuated DEN4 vector. Mutations identified in 5-FU DEN4 mutant viruses which were *ts* in only HuH-7 cells (Table 4) may potentially be utilized in vaccine candidates, such as rDEN4Δ30, to selectively control the replication and pathogenesis of DEN4 in the liver. These combined results from the sequence analysis of 5-FU mutant viruses demonstrate the utility of chemical mutagenesis as a means of introducing attenuating mutations into the dengue virus genome.

[0085] The presence of a point mutation at nt position 4,995 in eight separate mutant viruses was described above. Five additional point mutations were also represented in multiple viruses including nt changes at position 1,455 in E, 7,162, 7,163 and 7,564 in NS4B, and 10,275 in the 3' UTR (Table 5). The significance of the occurrence of these

"sister" mutations in multiple viruses is discussed in Example 6. Interestingly, the wild-type, parallel-passaged virus, 2A-13, also contained a single mutation at the 7,163 nt position in NS4B.

[0086] Introduction of a *ts* mutation into rDEN4 and rDEN4Δ30. The presence of a single nucleotide substitution (U > C mutation at nt position 4,995 in NS3) in three separate mutant viruses (clones 239, 489, and 773) indicated that this mutation specified the *ts* and *att* phenotypes in each of the three mutant viruses. This mutation was cloned into cDNA construct of p4 and p4Δ30 and recombinant viruses were recovered and designated rDEN4-4995 and rDEN4Δ30-4995, respectively. These recombinant viruses were tested for *ts* and *att* phenotypes as described above (Table 6). As expected, introduction of mutation 4995 into rDEN4 wt resulted in a significant *ts* phenotype at 39°C in both Vero and HuH-7 cells. rDEN4-4995 grew to nearly wild-type levels at the permissive temperature, 35°C, in both cell types, but demonstrated a greater than 10,000-fold reduction at 39°C (shut-off temperature) in both Vero and HuH-7 cells. The addition of the 4995 mutation to rDEN4Δ30 yields a recombinant virus, rDEN4Δ30-4995, that exhibits the same level of temperature sensitivity as rDEN4-4995 (Table 6).

[0087] The rDEN4 viruses encoding the 4995 mutation were next tested for replication in the brains of suckling mice (Table 6). The 4995 mutation conferred an *att* phenotype upon both rDEN4 and rDEN4Δ30. There was an approximately 1,000-fold reduction in virus replication compared to that of wt virus. The combination of point mutation 4995 and the Δ30 deletion did not appear to result in an additive reduction of virus replication. These results confirmed that the 4995 point mutation indeed specifies the *ts* and *att* phenotypes. Importantly, the utility of modifying tissue culture and *in vivo* phenotypes of the rDEN4Δ30 vaccine candidate by introduction of additional mutations was also demonstrated.

[0088] Discussion. Herein we teach how to prepare a tetravalent, live-attenuated dengue virus vaccine using rDEN4Δ30 as the DEN4 component and three antigenic chimeric viruses expressing the structural proteins (C, prM, and E) of DEN1, DEN2, and DEN3 from the attenuated rDEN4Δ30 vector (Example 8). DEN4 virus rDEN4Δ30 containing the Δ30 deletion mutation in the 3' UTR manifests restricted replication in humans while retaining

immunogenicity. Since rDEN4Δ30 retains a low level of residual virulence for humans despite this restricted replication, the present study was initiated to generate additional attenuating mutations that are envisioned as being useful to further attenuate rDEN4Δ30 or other dengue viruses and that are envisioned as being incorporated into any of the three antigenic chimeric viruses or other dengue viruses as needed. Temperature-sensitive mutants of dengue viruses (Bhamarapravati, N. & Yoksan, S. 1997 in: *Dengue and Dengue Hemorrhagic Fever* D.J. Gubler & G. Kuno eds. pp. 367-377 CAB International, New York; Eckels, K.H. *et al.* 1980 *Infect Immun* **27**:175-80) as well of other viruses (Skiadopoulos, M.H. *et al.* 1998 *J Virol* **72**:1762-8; Whitehead, S.S. *et al.* 1999 *J Virol* **73**:871-7) manifest restricted replication *in vivo*. We have generated a panel of 20 *ts* DEN4 mutant viruses, determined their genomic sequence, and assessed their *in vivo* attenuation phenotypes. The 20 *ts* DEN4 mutant viruses were generated by growth in the presence of 5-FU and were first selected for viability in Vero cells, the substrate planned for use in the manufacture of these vaccines, to ensure that the mutant viruses can be grown efficiently in a suitable substrate.

[0089] Two classes of mutant viruses were obtained; those *ts* in both Vero and HuH-7 cells ($n = 13$) or those *ts* in only HuH-7 cells ($n = 7$). The viruses exhibited a range in their level of temperature sensitivity from a 100- to 1,000,000-fold reduction in replication at the restrictive temperature of 39°C. Since our DEN4 vaccine candidate retains a low level of virulence for the liver and other findings support the ability of dengue viruses to infect hepatocytes (Lin, Y.L. *et al.* 2000 *J Med Virol* **60**:425-31; Marianneau, P. *et al.* 1997 *J Virol* **71**:3244-9) and cause liver pathology (Couvelard, A. *et al.* 1999 *Hum Pathol* **30**:1106-10; Huerre, M.R. *et al.* 2001 *Virchows Arch* **438**:107-15), we sought to develop mutations that would selectively restrict replication of dengue 4 virus in liver cells. Toward this end, we identified seven mutant viruses which have a HuH-7 cell-specific *ts* phenotype. The mutations present in these viruses are the first reported in DEN viruses that confer restricted replication in liver cells and are envisioned as being useful in limiting virus replication and pathogenesis in the liver of vaccine recipients. The contribution of individual mutations identified in the HuH-7 cell-specific *ts* viruses to the observed phenotypes is envisioned as being assessed by introduction of the individual mutations into recombinant DEN4 viruses.

[0090] Recent evidence has indicated that the magnitude of the viremia in DEN-infected patients positively correlates with disease severity, i.e., the higher the titer of viremia the more severe the disease (Murgue, B. *et al.* 2000 *J Med Virol* **60**:432-8; Vaughn, D.W. *et al.* 2000 *J Infect Dis* **181**:2-9). This indicates that mutations that significantly restrict replication of vaccine candidates *in vivo* are the foundation of a safe and attenuated vaccine. Evaluation of DEN virus vaccine candidates for *in vivo* attenuation is complicated by the lack of a suitable animal model which accurately mimics the disease caused by dengue viruses in humans. In the absence of such a model, the replication of the panel of 5-FU mutant viruses in the brains of Swiss Webster suckling mice was assessed as a means to identify an *in vivo* attenuation phenotype since this animal model is well-suited for the evaluation of a large set of mutant viruses. Each of the 20 *ts* mutant viruses exhibited an *att* phenotype manifesting a 10- to 6,000-fold reduction in replication in the brain of mice as compared to wt DEN4 virus (Table 2). This indicates that there is a correlation between the presence of the *ts* phenotype in tissue culture and attenuation of the mutant *in vivo* confirming the utility of selecting viruses with this marker as vaccine candidates. However, there was no correlation between the level of temperature sensitivity and the level of restriction *in vivo*. Furthermore, Sabin observed a dissociation between mouse neurovirulence and attenuation in humans by generating an effective live attenuated virus vaccine against DEN by passage of virus in mouse brain. This research actually resulted in a highly mouse-neurotropic DEN virus which, paradoxically, was significantly attenuated in humans (Sabin, A.B. 1952 *Am J Trop Med Hyg* **1**:30-50). Despite this, attenuation for the suckling mouse brain has been reported for other live-attenuated DEN virus vaccine candidates including the DEN2 PDK-53 vaccine strain which is non-lethal in mice and DEN-2 PR-159/S-1 vaccine strain which was significantly attenuated compared to its parental wild-type virus (Bhamarapravati, N. & Yoksan, S. 1997 in: *Dengue and Dengue Hemorrhagic Fever* D.J. Gubler & G. Kuno eds. pp. 367-377 CAB International, New York; Butrapet, S. *et al.* 2000 *J Virol* **74**:3011-9; Eckels, K.H. *et al.* 1980 *Infect Immun* **27**:175-80; Innis, B.L. *et al.* 1988 *J Infect Dis* **158**:876-80). Replication in rhesus monkeys has been reported to be predictive of attenuation for humans (Innis, B.L. *et al.* 1988 *J Infect Dis* **158**:876-80). Recently, murine models of DEN virus infection have been developed using SCID mice transplanted with human macrophage (Lin,

Y.L. *et al.* 1998 *J Virol* **72**:9729-37) or liver cell lines (An, J. *et al.* 1999 *Virology* **263**:70-7), but these mice have not as yet been used to assess *att* phenotypes of candidate vaccine viruses. Mutant viruses or recombinant viruses bearing one or more of these mutations described herein are envisioned as being tested for replication in rhesus monkeys (or other suitable animal model) as predictive for attenuation in humans.

[0091] The chemical mutagenesis of DEN4 virus and sequence analysis of resulting viruses described here has resulted in the identification of a large number of point mutations resulting in amino acid substitutions in all genes except C and NS4A as well as point mutations in the 5' and 3' UTR (Tables 3 and 4). This approach of whole-genome mutagenesis has the benefit of identifying mutations dispersed throughout the entire genome which are pre-selected for viability in the Vero cell substrate. Ten 5-FU mutant viruses which were *ts* in Vero and HuH-7 cells and three viruses which were selectively *ts* in HuH-7 cells contained only mutations outside of the genes encoding the structural proteins, i.e., in the 5' and 3' UTR or NS genes. These mutations along with the Δ30 deletion in the 3' UTR are particularly suited for inclusion in antigenic, chimeric vaccines which consist of an attenuated DEN4 vector bearing the wild-type structural genes (C, prM, E) of the other DEN virus serotypes. Use of this strategy has several advantages. Each antigenic chimeric virus that possesses structural proteins from a wild-type virus along with attenuating mutations in their UTRs or NS genes should maintain its infectivity for humans, which is mediated largely by the E protein, and, therefore, each vaccine component should be immunogenic (Huang, C.Y. *et al.* 2000 *J Virol* **74**:3020-8). The replicative machinery of the tetravalent vaccine strains would share the same attenuating mutations in the NS genes or in the UTR which should attenuate each vaccine component to a similar degree and thereby minimize interference or complementation among the four vaccine viruses. In addition, wild-type E protein would be expected to most efficiently induce neutralizing antibodies against each individual DEN virus.

[0092] Sequence analysis of dengue viruses (Blok, J. *et al.* 1992 *Virology* **187**:573-90; Lee, E. *et al.* 1997 *Virology* **232**:281-90; Puri, B. *et al.* 1997 *J Gen Virol* **78**:2287-91) and yellow fever viruses (Dunster, L.M. *et al.* 1999 *Virology* **261**:309-18; Holbrook, M.R. *et al.* 2000 *Virus Res* **69**:31-9) previously generated by serial passage in

tissue culture have mutations throughout much of the genome, a pattern we have observed in the present study. Recent analysis of the DEN2 PDK-53 vaccine strain has identified the important mutations involved in attenuation which were located in non-structural regions including the 5' UTR, NS1 and NS3 (Butrapet, S. *et al.* 2000 *J Virol* **74**:3011-9). This DEN2 vaccine strain has been used to generate a chimeric virus with DEN1 C-prM-E genes (Huang, C.Y. *et al.* 2000 *J Virol* **74**:3020-8). In separate studies, the sequence of the DEN1 vaccine strain 45AZ5 PDK-27 was determined and compared to parental viruses, but the mutations responsible for attenuation have not yet been identified (Puri, B. *et al.* 1997 *J Gen Virol* **78**:2287-91).

[0093] Several amino acid substitutions were identified in more than one *ts* 5-FU mutant virus (Table 5). Lee *et al.* have previously reported finding repeated mutations in separate DEN3 virus clones after serial passage in Vero cells (Lee, E. *et al.* 1997 *Virology* **232**:281-90). A mutation (K > N) identified in E at a.a. position 202 in a single DEN3 passage series was also found in our 5-FU mutant virus #1012 (K > E). Mutations observed in the 5-FU sister mutant viruses are envisioned as representing adaptive changes that confer an increased efficiency of DEN4 replication in Vero cells. Such mutations are envisioned as being beneficial for inclusion in a live-attenuated DEN virus vaccine by increasing the yield of vaccine virus during manufacture. Interestingly, three distinct amino acid substitutions were found in NS4B of the 5-FU sister mutant viruses. The exact function of this gene is unknown, but previous studies of live-attenuated yellow fever vaccines (Jennings, A.D. *et al.* 1994 *J Infect Dis* **169**:512-8; Wang, E. *et al.* 1995 *J Gen Virol* **76**:2749-55) and Japanese encephalitis vaccines (Ni, H. *et al.* 1995 *J Gen Virol* **76**:409-13) have identified mutations in NS4B associated with attenuation phenotypes.

[0094] The mutation at nt position 4995 of NS3 (S1632P) was present as the only significant mutation identified in three 5-FU mutant viruses (#239, #489, and #773). This mutation was introduced into a recombinant DEN4 virus and found to confer a *ts* and *att* phenotype (Table 6). These observations clearly identify the 4995 mutation as an attenuating mutation. Analysis of a sequence alignment (Chang, G.-J. 1997 in: *Dengue and Dengue Hemorrhagic Fever* D.J. Gubler & G. Kuno, eds. pp. 175-198 CAB International, New York) of the four dengue viruses indicated that the Ser at a.a. position 1632 is conserved in DEN1

and DEN2, while DEN3 contains an Asn at this position indicating that the mutation is predicted to be useful in modifying the phenotypes of the other DEN virus serotypes. The NS3 protein is 618 a.a. in length and contains both serine protease and helicase activities (Bazan, J.F. & Fletterick, R.J. 1989 *Virology* **171**:637-9; Brinkworth, R.I. *et al.* 1999 *J Gen Virol* **80**:1167-77; Valle, R.P. & Falgout, B. 1998 *J Virol* **72**:624-32). The 4995 mutation results in a change at a.a. position 158 in NS3 which is located in the N-terminal region containing the protease domain. Amino acid position 158 is located two a.a. residues away from an NS3 conserved region designated homology box four. This domain has been identified in members of the flavivirus family and is believed to be a critical determinant of the NS3 protease substrate specificity (Bazan, J.F. & Fletterick, R.J. 1989 *Virology* **171**:637-9; Brinkworth, R.I. *et al.* 1999 *J Gen Virol* **80**:1167-77). However, the exact mechanism which results in the phenotype associated with the 4995 mutation has not yet been identified. The identification of the 4995 mutation as an attenuating mutation permits a prediction of its usefulness for the further attenuation of rDEN4Δ30.

[0095] We have determined the contribution of individual 5-FU mutations to the observed phenotypes by introduction of the mutations into recombinant DEN4 viruses as was demonstrated herein for the 4995 mutation (see Example 3). In addition, combination of individual mutations with each other or with the Δ30 mutation is useful to further modify the attenuation phenotype of DEN4 virus candidate vaccines. The introduction of the 4995 mutation into rDEN4Δ30 described herein rendered the rDEN4Δ30-4995 double mutant *ts* and 1000-fold more attenuated for the mouse brain than rDEN4Δ30. This observation has demonstrated the feasibility of modifying both tissue culture and *in vivo* phenotypes of this and other dengue virus vaccine candidates. Once the mutations responsible for the HuH-7 cell-specific *ts* phenotype are identified as described above and introduced into the rDEN4Δ30 vaccine candidate, we envision confirming that these mutations attenuate rDEN4Δ30 vaccine virus for the liver of humans. A menu of attenuating mutations is envisioned as being assembled that is predicted to be useful in generating satisfactorily attenuated recombinant dengue vaccine viruses and in increasing our understanding of the pathogenesis of dengue virus (see Example 7).

Example 2

Chemical Mutagenesis of DEN4 Virus Results in Small-Plaque Mutant Viruses with Temperature-Sensitive and Attenuation Phenotypes

[0096] Mutations that restrict replication of dengue virus have been sought for the generation of recombinant live-attenuated dengue virus vaccines. Dengue virus type 4 (DEN4) was previously grown in Vero cells in the presence of 5-fluorouracil, and the characterization of 1,248 mutagenized, Vero cell-passaged clones identified 20 temperature-sensitive (*ts*) mutant viruses that were attenuated (*att*) in suckling mouse brain (Example 1). The present investigation has extended these studies by identifying an additional 22 DEN4 mutant viruses which have a small-plaque size (*sp*) phenotype in Vero cells and/or the liver cell line, HuH-7. Five mutant viruses have a *sp* phenotype in both Vero and HuH-7 cells, three of which are also *ts*. Seventeen mutant viruses have a *sp* phenotype in only HuH-7 cells, thirteen of which are also *ts*. Each of the *sp* viruses was growth restricted in the suckling mouse brain, exhibiting a wide range of reduction in replication (9- to 100,000-fold). Complete nucleotide sequence was determined for the 22 DEN4 *sp* mutant viruses, and nucleotide substitutions were found in the 3' untranslated region (UTR) as well as in all coding regions except NS4A. Identical mutations have been identified in multiple virus clones indicating that they are involved in the adaptation of DEN4 virus to efficient growth in Vero cells.

[0097] The DEN viruses cause more disease and death of humans than any other arbovirus, and more than 2.5 billion people live in regions with endemic dengue infection (Gubler, D.J. 1998 Clin Microbiol Rev **11**:480-96). Annually, there are an estimated 50-100 million cases of dengue fever (DF) and 500,000 cases of the more severe and potentially lethal dengue hemorrhagic fever / dengue shock syndrome (DHF/DSS) (Gubler, D.J. & Meltzer, M. 1999 Adv Virus Res **53**:35-70). Dengue fever is an acute infection characterized by fever, retro-orbital headache, myalgia, and rash. At the time of defervescence during DF, a more severe complication of DEN virus infection, DHF/DSS, may occur which is characterized by a second febrile period, hemorrhagic manifestations, hepatomegaly, thrombocytopenia, and hemoconcentration, which may lead to potentially life-threatening shock (Gubler, D.J. 1998 *Clin Microbiol Rev* **11**:480-96).

[0098] The sites of DEN virus replication in humans and their importance and relationship to the pathogenesis of DF and DHF/DSS are still incompletely understood (Innis, B.L. 1995 in: *Exotic Viral Infections* J.S. Porterfield, ed. pp. 103-146 Chapman and Hall, London). In addition to replication in lymphoid cells, it has become evident that the liver is involved in DEN infection of humans. Transient elevations in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels are observed in the majority of DEN virus-infected patients and hepatomegaly is observed in some patients (Kalayanarooj, S. *et al.* 1997 *J Infect Dis* **176**:313-21; Kuo, C.H. *et al.* 1992 *Am J Trop Med Hyg* **47**:265-70; Mohan, B. *et al.* 2000 *J Trop Pediatr* **46**:40-3; Wahid, S.F. *et al.* 2000 *Southeast Asian J Trop Med Public Health* **31**:259-63). DEN virus antigen-positive hepatocytes are seen surrounding areas of necrosis in the liver of fatal cases (Couvelard, A. *et al.* 1999 *Hum Pathol* **30**:1106-10; Huerre, M.R. *et al.* 2001 *Virchows Arch* **438**:107-15), from which dengue virus sequences were identified using RT-PCR (Rosen, L. *et al.* 1999 *Am J Trop Med Hyg* **61**:720-4). Of potential importance to the etiology of severe dengue virus infection, three studies have demonstrated that the mean levels of serum ALT and AST were significantly increased in patients with DHF/DSS compared to those with DF (Kalayanarooj, S. *et al.* 1997 *J Infect Dis* **176**:313-21; Mohan, B. *et al.* 2000 *J Trop Pediatr* **46**:40-3; Wahid, S.F. *et al.* 2000 *Southeast Asian J Trop Med Public Health* **31**:259-63). As expected, elevation of serum liver enzymes has previously been observed in clinical trials of DEN virus vaccine candidates (Example 8; Eckels, K.H. *et al.* 1984 *Am J Trop Med Hyg* **33**:684-9; Edelman, R. *et al.* 1994 *J Infect Dis* **170**:1448-55; Kanesa-thasan, N. *et al.* 2001 *Vaccine* **19**:3179-3188; Vaughn, D.W. *et al.* 1996 *Vaccine* **14**:329-36).

[0099] Based on the increasing disease burden associated with DEN virus infection over the past several decades, a vaccine which confers protection against the four dengue virus serotypes is needed, but none is presently licensed. Because of the increased risk for severe DHF/DSS associated with secondary infection with a heterologous DEN virus serotype (Burke, D.S. *et al.* 1988 *Am J Trop Med Hyg* **38**:172-80; Halstead, S.B. *et al.* 1977 *J Exp Med* **146**:218-29; Thein, S. *et al.* 1997 *Am J Trop Med Hyg* **56**:566-72), an effective vaccine must confer simultaneous protection against each of the four DEN virus serotypes. Several approaches are presently being pursued to develop a tetravalent vaccine against the

dengue viruses (Bancroft, W.H. *et al.* 1984 *J Infect Dis* **149**:1005-10; Bhamarapravati, N. & Sutee, Y. 2000 *Vaccine* **18**:44-7; Butrapet, S. *et al.* 2000 *J Virol* **74**:3011-9; Guirakhoo, F. *et al.* 2000 *J Virol* **74**:5477-85; Huang, C.Y. *et al.* 2000 *J Virol* **74**:3020-8; Kanesa-thasan, N. *et al.* 2001 *Vaccine* **19**:3179-3188). One such approach, a live-attenuated DEN4 vaccine candidate, termed 2AΔ30, was both attenuated and immunogenic in a cohort of 20 volunteers (Example 8). The recombinant 2AΔ30 virus contains a 30 nt deletion in the 3' UTR which removes nucleotides 10,478-10,507 and was found to produce a low or undetectable level of viremia in vaccinees at a dose of 10^5 PFU/vaccinee. An asymptomatic rash was reported in 50% of volunteers, and the only laboratory abnormality observed was an asymptomatic, transient rise in the serum ALT level in 5 of the 20 vaccinees. All 2AΔ30 vaccinees developed serum neutralizing antibodies against DEN4 virus (mean titer: 1:580), and 2AΔ30 was not transmitted to mosquitoes that fed experimentally on vaccinees (Troyer, J.M. *et al.* 2001 *Am J Trop Med Hyg* **65**:414-9). Because of the desirable properties conferred by the Δ30 mutation, chimeric vaccine candidates are being constructed which contain the structural genes of DEN virus type 1, 2, and 3, in the attenuated DEN4 background bearing the genetically stable Δ30 mutation. Attenuating mutations outside of the structural genes are particularly attractive for inclusion in antigenic chimeric vaccine candidates because they will not affect the infectivity or immunogenicity conferred by the major mediator of humoral immunity to DEN viruses, the envelope (E) protein.

[0100] The presence of rash and elevated ALT levels suggests that the 2AΔ30 vaccine candidate may be slightly under-attenuated in humans. Similarly, many previous attempts to develop live attenuated dengue virus vaccines have yielded vaccine candidates that were either over- or under-attenuated in humans, some of which also induced elevation of serum ALT and AST levels (Bhamarapravati, N. & Yoksan, S. 1997 in: *Dengue and Dengue Hemorrhagic Fever* D.J. Gubler & G. Kuno eds. pp. 367-377 CAB International, New York; Eckels, K.H. *et al.* 1984 *Am J Trop Med Hyg* **33**:684-9; Innis, B.L. *et al.* 1988 *J Infect Dis* **158**:876-80; Kanesa-thasan, N. *et al.* 2001 *Vaccine* **19**:3179-3188; McKee, K.T., Jr. *et al.* 1987 *Am J Trop Med Hyg* **36**:435-42). Therefore, we have developed a menu of point mutations conferring temperature-sensitive (*ts*), small-plaque (*sp*), and attenuation (*att*) phenotypes capable of attenuating DEN4 viruses to a varying degree (Example 1). We have

previously described 20 mutant viruses that exhibit a *ts*, but not *sp*, phenotype in Vero cells or HuH-7 liver cells and that show attenuated replication in mouse brain (Example 1). Addition of such mutations to 2AΔ30 or to other dengue virus vaccine candidates is envisioned as yielding vaccine candidates that exhibit a more satisfactory balance between attenuation and immunogenicity.

[0101] In the present Example, we have extended our analysis of the panel of 1,248 DEN4 virus clones previously generated by mutagenesis with 5-fluorouracil (5-FU) (Example 1), by identifying a set of 22 *sp* mutant viruses, some of which also have a *ts* phenotype. Small plaque mutant viruses were sought since such viruses are often attenuated in humans (Bhamarapravati, N. & Yoksan, S. 1997 in: *Dengue and Dengue Hemorrhagic Fever* D.J. Gubler & G. Kuno eds. pp. 367-377 CAB International, New York; Butrapet, S. *et al.* 2000 *J Virol* **74**:3011-9; Crowe, J.E.Jr. *et al.* 1994 *Vaccine* **12**:783-790; Crowe, J.E.Jr. *et al.* 1994 *Vaccine* **12**:691-699; Eckels, K.H. *et al.* 1980 *Infect Immun* **27**:175-80; Innis, B.L. *et al.* 1988 *J Infect Dis* **158**:876-80; Murphy, B.R. & Chanock, R.M. 2001 in: *Fields Virology* D.M. Knipe, *et al.* Eds. Vol. 1, pp. 435-468 Lippincott Williams & Wilkins, Philadelphia; Takemoto, K.K. 1966 *Prog Med Virol* **8**:314-48). Because natural infection with dengue viruses and vaccination with 2AΔ30 may be associated with liver toxicity in humans, we identified mutant viruses with restricted replication in human liver cells. Accordingly, viruses were screened for plaque size and temperature-sensitivity in the human hepatoma cell line, HuH-7, as well as in Vero cells. Here we describe the *ts* phenotype, nucleotide sequence, and growth properties in suckling mice of 22 *sp* DEN4 mutant virus clones.

[0102] **Cells and viruses.** WHO Vero cells (African green monkey kidney cells) and HuH-7 cells (human hepatoma cells) (Nakabayashi, H. *et al.* 1982 *Cancer Res* **42**:3858-63) were maintained as described in Example 1. DEN4 2A virus is a wild type virus derived from a cDNA clone of DEN4 strain 814669 (Dominica, 1981) (Lai, C.J. *et al.* 1991 *PNAS USA* **88**:5139-43; Mackow, E. *et al.* 1987 *Virology* **159**:217-28). The nucleotide sequence of DEN4 2A, the parent of the 5-FU mutant viruses, was previously assigned GenBank accession number AF375822 (Example 1). The DEN4 vaccine candidate, 2AΔ30, (Example 8) contains a 30 nt deletion in the 3' untranslated region (UTR) which removes nucleotides 10,478-10,507 (Men, R. *et al.* 1996 *J Virol* **70**:3930-7). The cDNA clones p4, a modified

derivative of the DEN4 2A cDNA clone, and p4Δ30 were used to generate recombinant wild type and attenuated viruses, rDEN4 and rDEN4Δ30, respectively (Example 8). GenBank accession numbers were previously assigned as follows (virus: accession number): DEN4 strain 814669: AF326573; 2AΔ30: AF326826; rDEN4: AF326825; rDEN4Δ30: AF326827.

[0103] Generation and biological cloning of mutant viruses with a *sp* phenotype. The generation of 1,248 virus clones from a pool of 5-fluorouracil-mutagenized DEN4 2A has been previously described (Example 1). Briefly, monolayers of Vero cells were infected with DEN4 2A at a multiplicity of infection (MOI) of 0.01 and overlaid with MEM supplemented with 2% FBS and 1 mM 5-fluorouracil (5-FU) (Sigma, St. Louis, MO), which reduced replication of DEN4 2A 100-fold. Vero cells in 96-well plates were inoculated with the 5-FU treated virus suspension, and virus clones were harvested from plates receiving terminally-diluted virus. A total of 1,248 virus clones were generated from the cultures treated with 1 mM 5-FU. Two virus clones, 2A-1 and 2A-13, were generated in the same manner from control cultures not treated with 5-FU and served as parallel-passaged control viruses with a wild type phenotype.

[0104] Evaluation of *in vitro* plaque size and temperature sensitivity. The 1,248 5-FU-mutagenized virus clones were screened for temperature sensitivity by assessing virus replication at 35°C (permissive temperature) and 39°C (restrictive temperature) in Vero and HuH-7 cells. Cell monolayers in 96-well plates were inoculated with serial ten-fold dilutions of virus and replicate plates were incubated at 35°C and 39°C for five days in temperature-controlled water baths. Virus replication was determined by immunoperoxidase staining as previously described (Example 1). A collection of 193 5-FU virus clones demonstrated a 100-fold or greater reduction in titer at 39°C in either cell line, and these presumptive *ts* viruses were further characterized. The efficiency of plaque formation (EOP) at permissive and restrictive temperatures and the plaque size of each of the 193 virus clones were determined as follows. Serial ten-fold dilutions of virus suspension were inoculated onto confluent Vero cell and HuH-7 cell monolayers in replicate 24-well plates. After incubation at 35°C for two hours, monolayers were overlaid with 0.8% methylcellulose (EM Science, Gibbstown, NJ) in L-15 medium (Quality Biologicals, Gaithersburg, MD) supplemented with 2% FBS, gentamicin, and L-glutamine. After incubation of replicate

plates for five days at 35, 37, 38, or 39°C in temperature-controlled water baths, plaques were visualized by immunoperoxidase staining and counted as previously described. Plaque size of each of the 193 viruses was evaluated at the permissive temperature (35°C) and compared to that of DEN4 2A-13 parallel-passaged control virus with a wild type plaque size. Mutant viruses incubated at the permissive temperature of 35°C which had a plaque size ≤1 mm or ≤0.4 mm (approximately ≤50% the size of wild type DEN4 2A-13) in Vero or HuH-7 cells, respectively, were designated as having a *sp* phenotype. The level of temperature sensitivity and plaque size of each virus was confirmed in at least two separate experiments. Seventy-five viruses which were confirmed to have a putative *ts* and/or *sp* phenotype were biologically cloned an additional two times and phenotypes were re-assessed. Twenty-two of the 75 terminally diluted viruses were found to have a *sp* phenotype. Sixteen of the 22 *sp* mutant viruses were also found to have a *ts* phenotype as defined by a 2.5 or 3.5 $\log_{10}$PFU/ml reduction in virus titer in Vero or HuH-7 cells, respectively, at restrictive temperature compared to the permissive temperature of 35°C as previously described (Example 1). Twenty of the 75 terminally-diluted viruses were found to have a *ts* phenotype without a *sp* phenotype and were previously described (Example 1). The remainder of the 75 viruses did not meet either criteria for a *ts* or *sp* mutant virus.

[0105] Evaluation of *sp* mutant viruses for restricted replication in suckling mice. Animal experiments were carried out in accordance with the regulations and guidelines of the National Institutes of Health, Bethesda, MD. Growth of DEN4 5-FU mutant viruses was determined in Swiss Webster suckling mice (Taconic Farms, Germantown, NY). Groups of six seven-day-old mice were inoculated intracerebrally with 10^4 PFU of virus in 30 µl Opti-MEM I (Invitrogen) and the brain of each mouse was removed five days later and individually analyzed as previously described (Example 1). Clarified supernatants of 10% suspensions of mouse brain were frozen at -70°C, and the virus titer was determined by plaque assay in Vero cells.

[0106] Determination of the complete genomic sequence of the *sp* mutant viruses. The nucleotide sequence of the 5-FU-mutagenized DEN4 viruses was determined as described in Example 8. Briefly, genomic RNA was isolated from virus clones and cDNA was prepared by reverse transcription and served as template for the generation of

overlapping PCR fragments. A panel of primers was designed to sequence both strands of the PCR product from which consensus sequences were assembled and analyzed. The nucleotide sequence of the 5' and 3' regions of the virus genome was determined after circularization of the RNA genome as described in Example 8.

[0107] Identification of DEN4 5-fluorouracil mutant viruses with a *sp* phenotype. The generation of a panel of 1,248 virus clones from a wild type DEN4 2A virus suspension mutagenized by 5-FU has been described previously (Example 1). In the present study twenty-two mutant viruses with a *sp* phenotype were identified. The plaque size of representative mutant viruses is illustrated in Figure 3. The plaque size of DEN4 2A-13 virus (a parallel-passaged virus with a wild type phenotype derived from control cultures not treated with 5-FU) was consistently smaller in HuH-7 cells than that observed in Vero cells (Figure 3A). Mutant viruses #569 and #1189 (Figure 3B) were *sp* in both Vero and HuH-7 cells. In contrast, 5-FU mutant virus clones #311 and #1083 (Figure 3C) were *sp* in only HuH-7 cells, suggesting a liver cell-specific defect in replication within this phenotypic group. As indicated in Table 7, five mutant viruses were found to have a *sp* phenotype in both Vero and HuH-7 cells while 17 viruses had a *sp* phenotype in only HuH-7 cells. Each 5-FU mutant virus clone was compared for a *sp* or *ts* phenotype with three control viruses, 2A-13, wild type rDEN4, and rDEN4Δ30. The recombinant viruses, rDEN4 and rDEN4Δ30, each had a plaque size in Vero and HuH-7 cells similar to that of DEN4 2A-13 indicating that the Δ30 mutation does not confer a *sp* phenotype (Table 7).

[0108] Most of the *sp* 5-FU mutant viruses also had a *ts* phenotype in Vero and/or HuH-7 cells (Table 7) since mutant viruses were initially screened for temperature sensitivity. Temperature-sensitivity was defined as a 2.5 or 3.5 $\log_{10}$PFU/ml reduction in virus titer in Vero or HuH-7 cells, respectively, at restrictive temperature compared to the permissive temperature of 35°C as previously defined (Example 1). Three mutant viruses (#574, #1269 and #1189) were *sp* and *ts* in both Vero and HuH-7 cells, while nine mutant viruses (#506-326 in Table 7) were found to be *ts* in both cell types but *sp* only in HuH-7 cells. Four viruses (#1104, 952, 738, and 1083) were found to have a wild type phenotype in Vero cells but were both *sp* and *ts* in HuH-7 cells. These four mutant viruses each had a 6,000- to 600,000-fold reduction in virus titer at 39°C in HuH-7 cells with only a 6- to 40-

fold reduction at 39°C in Vero cells. Finally, *sp* mutant viruses were identified which did not have a *ts* phenotype in either cell line; two of these viruses (#569 and #761) were *sp* in both Vero and HuH-7 cells and four viruses (#1096-1012) were *sp* in only HuH-7 cells (Table 7). As described previously, the Δ30 mutation did not confer temperature-sensitivity in either cell line (Example 1).

[0109] The *sp* 5-FU mutant viruses have restricted replication in suckling mouse brain. The 22 *sp* DEN4 5-FU mutant viruses were evaluated for their ability to replicate in the brain of one-week-old suckling mice. As a marker for *in vivo* attenuation, their level of replication was compared with that of the parallel-passaged control virus with a wild type phenotype, 2A-13 (Table 7). Nineteen of 22 *sp* mutant viruses had a greater than 100-fold reduction in virus replication in the brain of suckling mice compared to 2A-13 and nine viruses had a reduction of greater than 10,000-fold.

[0110] The five mutant viruses which were *sp* in both Vero and HuH-7 cells were 5,000-fold to 100,000-fold restricted in replication compared to 2A-13. Two of these mutant viruses, #569 and #761, were not *ts* in either cell line but had a reduction in virus titer of greater than 10,000-fold in mouse brain, indicating that the *sp* phenotype in both Vero and HuH-7 cells is an important surrogate marker for attenuated replication in suckling mouse brain. 5-FU mutant viruses which were *sp* in only HuH-7 cells had a more variable range of replication in mouse brain. Three viruses had a mean reduction in virus titer of less than 10-fold when compared to 2A-13 virus. However, 8 of 13 viruses which were *ts* in Vero and/or HuH-7 cells but *sp* in only HuH-7 cells had a greater than 5,000-fold reduction in virus replication. The results of the *in vivo* replication analysis of the previously described 20 *ts* 5-FU mutant viruses (Example 1) and the 22 *sp* mutant viruses are summarized in Table 8. Mutant viruses with both a *sp* and *ts* phenotype were found to have a significantly greater level of attenuation in the brain of suckling mice when compared to viruses with only a *ts* phenotype.

[0111] Sequence analysis of the *sp* 5-FU mutant viruses. To initiate an analysis of the genetic basis of the *ts*, *sp*, or *att* phenotype of the 22 *sp* mutant viruses, the complete nucleotide sequence of each virus genome was determined and is summarized in Table 9 (*sp* in Vero and HuH-7 cells) and Table 10 (*sp* in only HuH-7 cells). All identified

mutations were nucleotide substitutions, as deletions or insertions were not observed. Point mutations were distributed throughout the genome, including the 3' UTR as well as in all coding regions. Because all 5-FU mutant viruses were found to have at least two mutations (two to six), the observed phenotypes cannot be directly attributed to a specific mutation. The majority of *sp* viruses also contained translationally silent point mutations (none to four) in the structural or non-structural coding regions. However, these silent mutations are not expected to contribute to the observed phenotypes. Six of the 22 *sp* mutant viruses (Tables 9 and 10) were found to have mutations in only the NS genes and/or the 3' UTR, indicating that the *sp* phenotype can be conferred by mutations outside of the structural genes.

[0112] Presence of identical mutations in multiple 5-FU mutant viruses. Analysis of the complete nucleotide sequence data for the 5-FU mutant viruses identified several repeated mutations which were present in two or more viruses. Such mutations were also identified previously during our analysis of twenty 5-FU mutant viruses with a *ts* but not *sp* phenotype (Example 1). Because these mutations occurred in viruses together with additional mutations, the contribution of the repeated mutations to the observed *sp*, *ts*, and *att* phenotypes remains empirical. Table 11 lists the repeated mutations found among the 20 *ts* (not *sp*) mutant viruses described previously (Example 1) and the 22 *sp* mutant viruses described here. Repeated mutations were identified in the following genes: two in E, two in NS3, five in NS4B, one in NS5, and two in the 3' UTR. Interestingly, within a thirty nucleotide region of NS4B (nt 7153-7182), there were five different nucleotide substitutions which were found in sixteen viruses. Also at nt 7,546 in NS4B, an amino acid substitution (Ala → Val) was found in 10 different 5-FU mutant viruses. The significance of these repeated mutations in NS4B as well as in other DEN4 genomic regions remains empirical, but a reasonable explanation for this phenomenon is that these mutations are involved in adaptation of DEN4 virus for efficient growth in Vero cells, as further discussed in Example 6.

[0113] Discussion. As part of a molecular genetic vaccine strategy, we have developed attenuating mutations that are envisioned as being useful in the development of a live attenuated tetravalent dengue virus vaccine. Specifically, mutations which restrict replication of the vaccine virus in human liver cells were generated since there was some

residual virulence of the rDEN4Δ30 vaccine candidate for the liver of humans. Mutant viruses with a *sp* phenotype were sought in both Vero cells and HuH-7 human liver cells, in order to identify host-range mutant viruses that were specifically restricted in replication in HuH-7 cells (*sp* in HuH-7 but not in Vero). Such mutations are envisioned as being useful in limiting replication of a candidate vaccine in the liver of vaccinees while preserving both efficient replication in Vero cells and immunogenicity *in vivo*.

[0114] Several observations from the present study indicate that *sp* mutations confer an *att* phenotype *in vivo*. This is not surprising since attenuation in suckling mouse brain has been reported for live DEN virus vaccine candidates possessing *sp* phenotypes, including the DEN2 PDK-53 and DEN2 PR-159/S-1 vaccine strains (Bhamarapravati, N. & Yoksan, S. 1997 in: *Dengue and Dengue Hemorrhagic Fever* D.J. Gubler & G. Kuno eds. pp. 367-377 CAB International, New York; Butrapet, S. *et al.* 2000 *J Virol* **74**:3011-9; Eckels, K.H. *et al.* 1980 *Infect Immun* **27**:175-80; Innis, B.L. *et al.* 1988 *J Infect Dis* **158**:876-80). Each of 22 DEN4 5-FU mutant viruses with a *sp* phenotype (some of which were also *ts*) in either Vero or HuH-7 cells manifested restricted replication in the brains of mice. Six 5-FU mutant viruses with a *sp* phenotype in the absence of a *ts* phenotype were more attenuated in the brains of suckling mice than mutant viruses with solely a *ts* phenotype (Example 1), indicating that the *sp* phenotype specifies a greater level of attenuation for mouse brain than does the *ts* phenotype. Mutant viruses with both a *ts* and *sp* phenotype had an even greater reduction in replication, further indicating that the attenuation conferred by the *ts* and *sp* phenotypes can be additive. Importantly, seventeen of the 22 *sp* mutant viruses were host-range *sp* mutant viruses, being *sp* only in HuH-7 cells. Since such mutations are envisioned as being useful in restricting the replication of a DEN4 virus in human liver cells, we used nucleotide sequence analysis to determine the genetic basis of the *sp* phenotype.

[0115] Analysis of the complete genomic sequence of the 22 *sp* DEN4 viruses revealed substitutions in the 3' UTR as well as coding mutations in all genes except NS4A. It was first noted that several specific mutations were present in two or more of the 22 *sp* DEN4 mutant viruses and that many of these same mutations were also previously identified among the set of 20 *ts* DEN4 mutant viruses (Example 1). Since flaviviruses can rapidly accumulate mutations during passage in tissue culture (Dunster, L.M. *et al.* 1999 *Virology*

261:309-18; Mandl, C.W. *et al.* 2001 *J Virol* **75**:5627-37), many of these over-represented mutations, previously referred to as putative Vero cell adaptation mutations (Example 1), likely promote efficient replication in Vero cells and were selected unintentionally during the biological cloning of the mutant viruses. The effect of these mutations on DEN virus replication in Vero cells, the proposed substrate for vaccine manufacture, is discussed in Example 6.

[0116] The *sp* mutations identified among the 5-FU mutant viruses are envisioned as being useful in several different approaches for the development of DEN virus vaccine strains. As described above for the generation of antigenic chimeric viruses, one or more *sp* attenuating mutations are envisioned as being added to the attenuated DEN4Δ30 genetic background to supplement the *att* phenotype of the Δ30 mutation. A second approach is to introduce a *sp* attenuating mutation, with or without Δ30, into infectious cDNA clones of the other three DEN serotypes. The ability to transfer mutations among genetically-related viruses and maintain similar *att* phenotypes has been previously demonstrated (Skiadopoulos, M.H. *et al.* 1999 *Virology* **260**:125-35). These distinct strategies are envisioned as being useful as separate or complementary approaches to the construction of a tetravalent DEN virus vaccine, underlining the importance of the identification of a large panel of *att* mutations within the DEN viruses.

Example 3

Recombinant DEN4 Viruses Containing Mutations Identified in 5-FU Mutant Viruses show Restricted Replication in Suckling Mouse Brain and in SCID Mice Transplanted with Human Liver Cells

[0117] Data was presented in Examples 1 and 2 that summarizes the generation, characterization and sequence analysis of 42 attenuated mutant DEN4 viruses. For three of the mutant viruses (#239, 489, and 773) with a single missense mutation at nt position 4995 in NS3, it was clear that the identified mutation specified the *ts* and *att* phenotypes. This conclusion was confirmed in Example 1 by tissue culture and *in vivo* characterization of rDEN4-4995, a recombinant virus into which the 4995 mutation had been introduced by site-directed mutagenesis. In this analysis, rDEN4-4995 exhibited the same level of temperature sensitivity and attenuation as 5-FU mutant viruses #239, 489, and 773. The individual

mutation(s) in the remaining 5-FU mutant viruses that specify the observed phenotypes remains to be identified, since most of these viruses possess more than one nucleotide substitution. We have conducted an analysis to identify the mutations in a subset of the other 39 mutant viruses that specify the *ts*, *sp*, and *att* phenotypes by introduction of each mutation into the wt DEN4 cDNA (p4) and evaluation of the phenotypes of the resulting recombinant DEN4 viruses bearing the individual mutations. Previous studies of a DEN2 virus vaccine candidate (Butrapet, S. *et al.* 2000 *J Virol* **74**:3011-9) as well as other virus vaccines (Whitehead, S.S. *et al.* 1999 *J Virol* **73**:871-7) have demonstrated the utility of this approach for the identification of the genetic basis of attenuation.

[0118] As described in Examples 1 and 2, 19 5-FU mutant viruses were identified which were found to contain coding mutations in only the NS genes and/or nucleotide substitutions in the 5' or 3' UTR which would facilitate the generation of antigenic chimeric viruses. In the present example, the genetic basis of the observed *sp*, *ts*, and mouse brain *att* phenotypes was identified for these 19 viruses using reverse genetics to generate recombinant DEN4 (rDEN4) viruses containing individual mutations identified in the panel of DEN4 mutant viruses. In addition, the 19 5-FU mutant viruses were evaluated for replication in a novel small animal model for DEN4 virus replication, SCID mice transplanted with HuH-7 cells (SCID-HuH-7), and the genetic basis of the *att* viruses was identified using mutant rDEN4 viruses. Also presented are findings describing the generation and characterization of a recombinant virus containing two of the identified attenuating mutations as well as combination of select 5-FU mutations with the Δ30 mutation.

[0119] **Generation of rDEN4 viruses containing 5-FU mutations.** The methods used for the generation of rDEN4 viruses are outlined in Figure 4 and are similar to those described in Example 1. Briefly, the p4 cDNA was digested with the appropriate restriction enzymes and the resulting fragments were subcloned into a modified pUC119 vector. For Kunkel mutagenesis, single-stranded DNA preparations of the pUC-NS vectors were made, and primers were designed to individually introduce mutations that were present in the 5-FU mutant viruses. The sequences of the 41 mutagenic oligonucleotides used to generate the single-mutation recombinant viruses are presented in Table 12. Primers were designed to co-introduce or co-ablate a translationally-silent restriction enzyme site in the

cDNA, which greatly facilitates the screening and identification of cDNA clones possessing the mutant sequence. Fragments containing the introduced mutations were cloned back into p4, and nucleotide sequence analysis confirmed the presence of the nucleotide changes. A total of 33 rDEN4 viruses was generated which contained each of the individual mutations present in the 19 5-FU mutant viruses containing only coding mutations in the NS genes and/or nucleotide substitutions in the 5' or 3' UTR. An additional 8 rDEN4 viruses were generated from mutations identified in the remaining panel of 42 5-FU mutant viruses.

[0120] A cDNA clone was also generated which combined the mutations identified at nt position 4995 in NS3 and 7849 in NS5. The 7849 mutation was introduced into the p4-4995 cDNA clone by replacing the *Xma*I – *Pst*I fragment with that derived from the p4-7849 cDNA clone. The presence of both mutations was confirmed by sequence analysis. The Δ30 mutation was introduced into the 3' UTR of the individual mutant cDNA clones by replacing the *Mlu*I – *Kpn*I fragment with that derived from the p4Δ30 cDNA clone, and the presence of the deletion was confirmed by sequence analysis.

[0121] Recombinant viruses were recovered by transfection of Vero or C6/36 cells with RNA transcripts derived from the mutant cDNA clones as described in Example 1. Recovered viruses were terminally diluted twice and working stocks of viruses were prepared in Vero cells. Each of the mutant cDNA clones was recovered after transfection as expected since the 5-FU mutant viruses containing these mutations were viable.

[0122] **Characterization of *ts* and *att* phenotypes of the rDEN4 viruses containing introduced mutations.** Of the 19 5-FU mutant viruses with mutations in only NS genes and/or the 5' or 3' UTR, six had an *sp* phenotype (Table 13), ten had a *ts* phenotype in Vero and HuH-7 cells (Table 14), and three had a *ts* phenotype in only HuH-7 cells (Table 15). For the six *sp* 5-FU mutant viruses, #738, 922, 1081, 1083, 1136, and 1189, seventeen mutations identified by sequence analysis resulted in a coding change or a nucleotide change in the UTR and each was engineered into an individual DEN4 cDNA clone. Virus containing each defined mutation was successfully recovered and propagated and was tested for efficiency of plaque formation in Vero and HuH-7 cells at various temperatures, plaque size phenotype, and growth properties in suckling mice using methods previously described in Examples 1 and 2.

[0123] Table 13 lists the phenotypes of the six *sp* 5-FU mutant parent viruses and those of the 17 rDEN4 viruses encoding single mutations present in the parent virus. For example, 5-FU mutant #1189 (parent), which was *ts* and *sp* in both cell lines and had an almost 10,000-fold reduction in replication in suckling mouse brain, contained 4 coding mutations at nt position 3303 in NS1, 4812 and 5097 in NS3, and 7182 in NS4B. Analysis of the four rDEN4 viruses containing each of these mutations indicated that rDEN4-5097 had a *ts*, *sp*, and *att* phenotype while rDEN4-3303, rDEN4-4812, and rDEN4-7182 had no discernible phenotypes, indicating that the mutation at nt 5097 was responsible for the phenotype observed in the 5-FU parent, #1189. Thus, analysis of the relative contributions of the four mutations present in the 5-FU mutant #1189 to its attenuation phenotype provides the framework for a similar analysis of the remaining 5-FU mutant viruses. This analysis specifically demonstrates the methods used to identify mutations contributing to the observed phenotype. The *ts*, *sp*, and *att* phenotypes of 5-FU parent viruses #738, 922, 1081, and 1083, were similarly attributed to single mutations 3540, 4306, 2650, and 10634, respectively. However, two separate mutations (3771 and 4891) contributed to the phenotypes of 5-FU mutant virus #1136.

[0124] Table 14 lists the genetic basis of the *ts* and mouse brain attenuation for the ten 5-FU mutant viruses with *ts* phenotypes in both Vero and HuH-7 cells. As described in Example 1, the 4995 mutation which is the only mutation present in three 5-FU mutant viruses, #239, #489, and #773, was found to confer a *ts* and *att* phenotype, confirming the genetic basis for the phenotypes exhibited by these viruses. In three separate experiments, the rDEN4-4995 virus was found to have an approximately 1,000-fold decrease in replication in the brains of suckling mice when compared to that of wild-type virus (Table 6 and 14). The 4995 mutation is also present in 5-FU mutant viruses #473, #759, and #816, each of which has additional mutations. The *ts* and *att* phenotypes observed in these viruses can be attributed to the 4995 mutation since the additional mutations did not show discernible phenotypes. Interestingly, 5-FU mutant virus #938 has the 4995 mutation and an additional mutation at nt 3442 in NS1 with both mutations independently conferring restricted replication in mouse brain. The remaining three 5-FU parent viruses in Table 14, #173,

#509, and #1033, were found to each contain a single mutation responsible for the *att* phenotype: 7849, 8092, and 4907, respectively.

[0125] Three 5-FU mutant viruses, #686, #992, and #1175 with HuH-7 cell-specific *ts* phenotypes are listed in Table 15. Mutations in NS3 (5695) and NS5 (10186) were found to confer the phenotypes observed for parent virus #992 and #1175. Interestingly, two mutations in NS2A, 3575 and 4062, were found to result in a synergistic increase in the level of attenuation. Both individual mutations had an approximately 100-fold decrease in virus replication in the brain while the parent virus with both mutations had an almost 10,000-fold reduction. Table 16 lists two additional mutations with an *att* phenotype, 4896 and 6259 in NS3.

[0126] **Replication of DEN4 viruses in SCID mice transplanted with HuH-7 cells.** Since DEN viruses replicate poorly in the liver of mice and corresponding studies are impractical to conduct in non-human primates, an animal model that evaluates the *in vivo* level of replication of DEN virus in liver cells was developed based on a recent report examining the replication of DEN virus in SCID mice transplanted with a continuous cell line of human liver tumor cells (An, J. *et al.* 1999 *Virology* **263**:70-7). SCID mice transplanted with human continuous cell lines, primary cells, or organized tissues have similarly been used to study the replication of other viruses which lack a suitable small animal model (Mosier, D.E. 2000 *Virology* **271**:215-9). In our study, SCID mice were transplanted with HuH-7 cells since DEN4 virus replicated efficiently in these cells in tissue culture and since these were the cells used to define the host-range phenotype. These studies are envisioned as addressing the utility of examining DEN virus infection in SCID mouse-xenograft models for vaccine development (An, J. *et al.* 1999 *Virology* **263**:70-7; Lin, Y.L. *et al.* 1998 *J Virol* **72**:9729-37).

[0127] To further examine the *in vivo* growth properties of the 19 5-FU mutant DEN4 viruses with mutations in only the NS genes and/or the 3' UTR and selected corresponding rDEN4 mutant viruses, replication was assessed in SCID mice transplanted with HuH-7 cells (SCID-HuH-7). For analysis of DEN4 virus replication in SCID-HuH-7 mice, four to six week-old SCID mice (Tac:Icr:Ha(ICR)-*Prkdc*^{scid}) (Taconic Farms) were injected intraperitoneally with 10^7 HuH-7 cells suspended in 200 µl phosphate-buffered

saline (PBS). In preparation for transplantation, HuH-7 cells were propagated in cell culture as described above and harvested by trypsinization at approximately 80% confluence. Cells were washed twice in PBS, counted, resuspended in an appropriate volume of PBS, and injected into the peritoneum of mice. Tumors were detected in the peritoneum five to six weeks after transplantation, and only mice with apparent tumors were used for inoculation. Mice were infected by direct inoculation into the tumor with 10^4 PFU of virus in 50 µl Opti-MEM I. Mice were monitored daily for seven days and serum for virus titration was obtained by tail-nicking on day 6 and 7. Approximately 400 µl blood was collected in a serum separator tube (Sarstedt, Germany), centrifuged, and serum was aliquoted and stored at –70°C. The virus titer was determined by plaque assay in Vero cells. Seven days after infection, most mice developed morbidity and all mice were sacrificed. Tumors were excised and weighed to confirm uniformity of the experimental groups.

[0128] Preliminary experiments indicated that SCID-HuH-7 mice inoculated with DEN4 2A-13 directly into the tumor developed viremia with maximum levels (up to 8.0 $\log_{10}$PFU/ml serum) achieved on day 5 (Table 17). Virus could also be detected in brain, liver, and tumor homogenates.

[0129] The level of viremia in SCID-HuH-7 mice infected with parental 5-FU or rDEN4 mutant viruses was compared with that of the parallel-passaged control virus, 2A-13, or rDEN4, respectively. Results of 4 separate experiments indicated that the vaccine candidate, rDEN4Δ30, had an almost 10-fold reduction in virus replication compared to wild type rDEN4 (Table 13) which reflects the apparent attenuation of the rDEN4Δ30 vaccine candidate in humans (Example 8). Results in Tables 13 to 15 indicate that three 5-FU mutant viruses had a greater than 100-fold reduction in viremia in the SCID-HuH-7 mice compared to wild type 2A-13 virus: #1081, #1083, and #1189. The common phenotype among these viruses was a *sp* phenotype in HuH-7 cells. Analysis of the genetic basis of the *att* phenotype in these parent 5-FU mutant viruses identified three individual mutations in NS1, NS3, and the 3' UTR which conferred at least a 100-fold reduction in viremia. Specifically, rDEN4-2650 (NS1), rDEN4-5097 (NS3), and rDEN4-10634 (3' UTR) manifested a 2.2, 3.6, and 4.3 $\log_{10}$PFU/ml reduction in peak titer of viremia compared to rDEN4, respectively. These mutations also conferred the *att* phenotype in suckling mouse brain. 5-FU mutant virus #738

and #509 had a reduction in viremia in the SCID-HuH-7 mice compared to wild type 2A-13 of 1.9 and 1.5 $\log_{10}$PFU/ml, respectively, and the genetic basis for these phenotypes is envisioned as being assessed on an empirical basis.

[0130] This analysis of the genetic basis of the phenotypes specified by the mutations in the 5-FU mutant viruses that manifested restricted replication in SCID-HuH-7 mice indicated that (1) three separate mutations conferred the *att* phenotype; (2) these mutations were located in two proteins, NS1 and NS3, and in the 3' UTR; (3) these three mutations were fully responsible for each of the cell culture (*ts* or *sp*) and *in vivo* (attenuation in mouse brain and SCID-HuH-7 mice) phenotypes of the parent viruses; and (4) two of the three mutations specify the host-range *sp* phenotype (*sp* on HuH-7 only) and therefore are envisioned as being useful in a vaccine virus. Although the relevance of such SCID-transplant models to virus replication and disease in humans is unknown, the identification of three novel mutations which restrict DEN4 virus replication in SCID-HuH-7 mice is envisioned as facilitating an examination of the correlation between the *att* phenotype in SCID-HuH-7 mice with that in rhesus monkeys or humans. Such mutations, specifically the host-range *sp* mutations, are envisioned as being useful in conjunction with the Δ30 or other mutation to decrease the residual virulence of rDEN4Δ30 or other dengue virus for the human liver, and studies are envisioned as being conducted to construct such rDEN4 viruses and evaluate them in monkeys and humans (Example 8).

[0131] **Combination of two 5-FU mutations results in an additive *ts* phenotype.** The ability to combine individual mutations in rDEN4 virus as a means to modulate the phenotype of the resulting double mutant virus is a major advantage of using recombinant cDNA technology to generate or modify dengue virus vaccine candidates. Addition of multiple *ts* and *att* mutations to recombinant vaccine viruses is envisioned as improving the phenotypic stability of the double recombinant due to the decreased possibility of co-reversion of the two mutations to wild-type virulence (Crowe, J.E.Jr. *et al.* 1994a *Vaccine* **12**:783-790; Skiadopoulos, M.H. *et al.* 1998 *J Virol* **72**:1762-8; Subbarao, E.K. *et al.* 1995 *J Virol* **69**:5969-5977; Whitehead, S.S. *et al.* 1999 *J Virol* **73**:871-7). The mutations identified at nt position 4995 in NS3 and 7849 in NS5 were combined in a single p4 cDNA clone and a recombinant virus, designated rDEN4-4995-7849, was recovered and evaluated

for its *ts* and *att* phenotypes (Table 18). rDEN4-4995-7849 was more *ts* than either recombinant virus containing the individual mutations (Table 18), indicating the additive effect of the two *ts* mutations. The rDEN4-4995-7849 virus had a greater than 10,000-fold reduction in replication in the brains of suckling mice. The reduction in replication of the double mutant virus was only slightly increased over that of rDEN4-7849, however, a difference in the level of replication between rDEN4-4995-7849 and rDEN4-7849 would be difficult to detect since the level of replication of both viruses was close to the lower limit of detection (2.0 $\log_{10}$PFU/g brain).

[0132] Combination of selected 5-FU mutations with the Δ30 mutation confers increased attenuation of rDEN4Δ30 for the brains of suckling mice. To define the effect of adding individual mutations to the attenuated rDEN4Δ30 background, five combinations have been constructed: rDEN4Δ30-2650, rDEN4Δ30-4995, rDEN4Δ30-5097, rDEN4Δ30-8092, and rDEN4Δ30-10634. Addition of such missense mutations with various *ts*, *sp*, and *att* phenotypes is envisioned as serving to decrease the reactogenicity of rDEN4Δ30 while maintaining sufficient immunogenicity.

[0133] The Δ30 mutation was introduced into the 3' UTR of the individual mutant cDNA clones by replacing the *Mlu*I – *Kpn*I fragment with that derived from the p4Δ30 cDNA clone, and the presence of the deletion was confirmed by sequence analysis. Recombinant viruses were recovered by transfection in C6/36 cells for each rDEN4 virus. However, upon terminal dilution and passage, the rDEN4Δ30-5097 virus was found to not grow to a sufficient titer in Vero cells and was not pursued further. This is an example of a cDNA in which the 5-FU mutation and the Δ30 mutation are not compatible for efficient replication in cell culture. To begin the process of evaluating the *in vivo* phenotypes of the other four viruses which replicated efficiently in cell culture, rDEN4 viruses containing the individual mutations and the corresponding rDEN4Δ30 combinations were tested together for levels of replication in suckling mouse brain. The results in Table 19 indicate that addition of each of the mutations confers an increased level of attenuation in growth upon the rDEN4Δ30 virus, similar to the level conferred by the individual 5-FU mutation. No synergistic effect in attenuation was observed between the missense mutations and Δ30. These results indicate that the missense mutations at nucleotides 2650, 4995, 8092, and 10634 are compatible with

Δ30 for growth in cell culture and *in vivo* and can further attenuate the rDEN4Δ30 virus in mouse brain. Further studies in SCID-HuH-7 mice, rhesus monkeys, and humans are envisioned as establishing the effect of the combination of individual mutations and Δ30 upon attenuation and immunogenicity (Example 8).

[0134] By identifying the specific mutations in the 5-FU mutant viruses which confer the observed phenotypes, a menu of defined *ts*, *sp*, and *att* mutations is envisioned as being assembled (see Example 7). Numerous combinations of two or more of these mutations are envisioned as being selected with or without the Δ30 mutation. Such mutations and their combinations are envisioned as being useful for the construction of recombinant viruses with various levels of *in vivo* attenuation, thus facilitating the generation of candidate vaccines with acceptable levels of attenuation, immunogenicity, and genetic stability.

Example 4

Generation of DEN4 Mutant Viruses with Temperature-Sensitive and Mouse Attenuation Phenotypes Through Charge-Cluster-to-Alanine Mutagenesis

[0135] The previous Examples described the creation of a panel of DEN4 mutant viruses with *ts*, *sp*, and *att* phenotypes obtained through 5-FU mutagenesis. As indicated in these Examples, the attenuating mutations identified in the 5-FU mutant viruses are envisioned as having several uses including (1) fine tuning the level of attenuation of existing dengue virus vaccine candidates and (2) generation of new vaccine candidates by combination of two or more of these attenuating mutations. In the current example, we created a second panel of mutant viruses through charge-cluster-to-alanine mutagenesis of the NS5 gene of DEN4 and examined the resulting mutant viruses for the *ts*, *sp*, and *att* phenotypes as described in Examples 1 and 2. The charge-cluster-to-alanine mutant viruses recovered demonstrated a range of phenotypes including *ts* in Vero cells alone, *ts* in HuH-7 cells alone, *ts* in both cell types, *att* in suckling mouse brains, and *att* in SCID-HuH-7 mice.

[0136] The usefulness of mutant viruses expressing these phenotypes has already been described, however charge-cluster-to-alanine mutant viruses possess some additional desirable characteristics. First, the relevant mutations are envisioned as being designed for use in the genes encoding the non-structural proteins of DEN4, and therefore are envisioned

as being useful to attenuate DEN1, DEN2, and DEN3 antigenic chimeric recombinants possessing a DEN4 vector background. Second, the phenotype is usually specified by three or more nucleotide changes, rendering the likelihood of reversion of the mutant sequence to that of the wild type sequence less than for a single point mutation, such as mutations identified in the panel of 5-FU mutant viruses. Finally, charge-cluster-to-alanine attenuating mutations are envisioned as being easily combinable among themselves or with other attenuating mutations to modify the attenuation phenotype of DEN4 vaccine candidates or of DEN1, DEN2, and DEN3 antigenic chimeric recombinant viruses possessing a DEN4 vector background.

[0137] Charge-Cluster-to-Alanine-Mutagenesis. The cDNA p4, from which recombinant wild type and mutant viruses were generated, has been described in Examples 1, 2, and 3 and in Figure 4. Charge-cluster-to-alanine mutagenesis (Muylaert, I.R. *et al.* 1997 *J Virol* **71**:291-8), in which pairs of charged amino acids are replaced with alanine residues, was used to individually mutagenize the coding sequence for 80 pairs of contiguous charged amino acids in the DEN4 NS5 gene. Subclones suitable for mutagenesis were derived from the full length DEN4 plasmid (p4) by digestion with *Xma*I/*Pst*I (pNS5A), *Pst*I/*Sac*II (pNS5B) or *Sac*II/*Mlu*I (pNS5C) at the nucleotide positions indicated in Figure 4. These fragments were then subcloned and Kunkel mutagenesis was conducted as described in Examples 1 and 3. To create each mutation, oligonucleotides were designed to change the sequence of individual pairs of codons to GCAGCX (SEQ ID NO: 69), thereby replacing them with two alanine codons (GCX) and also creating a *Bbv*I restriction site (GCAGC) (SEQ ID NO: 70). The *Bbv*I site was added to facilitate screening of cDNAs and recombinant viruses for the presence of the mutant sequence. Restriction enzyme fragments bearing the alanine mutations were cloned back into the full-length p4 plasmid as described in Examples 1 and 3.

[0138] Initial evaluation of the phenotype of the 32 charge-cluster-to-alanine mutant viruses revealed a range in restriction of replication in suckling mouse brain and SCID-HuH-7 mice. To determine whether attenuation could be enhanced by combining mutations, double mutant viruses carrying two pairs of charge-cluster-to-alanine mutations were created by swapping appropriate fragments carrying one pair of mutations into a

previously-mutagenized p4 cDNA carrying a second pair of mutations in a different fragment using conventional cloning techniques.

[0139] **Transcription and Transfection.** 5'-capped transcripts were synthesized *in vitro* from mutagenized cDNA templates using AmpliCap SP6 RNA polymerase (Epicentre, Madison, WI). Transfection mixtures, consisting of 1 μg of transcript in 60 μl of HEPES/saline plus 12 μl of dioleoyl trimethylammonium propane (DOTAP) (Roche Diagnostics Corp., Indianapolis, IN), were added, along with 1 ml Virus production-serum free medium (VP-SFM) to subconfluent monolayers of Vero cells in 6-well plates. Transfected monolayers were incubated at 35°C for approximately 18 hr, cell culture medium was removed and replaced with 2 ml VP-SFM, and cell monolayers were incubated at 35°C. After 5 to 6 days, cell culture medium was collected, and the presence of virus was determined by titration in Vero cells followed by immunoperoxidase staining as previously described. Recovered virus was amplified by an additional passage in Vero cells, and virus suspensions were combined with SPG (sucrose--phosphate--glutamate) stabilizer (final concentration: 218 mM sucrose, 6 mM L-glutamic acid, 3.8 mM potassium phosphate, monobasic, and 7.2 mM potassium phosphate, dibasic, pH 7.2), aliquoted, frozen on dry ice, and stored at –70°C.

[0140] cDNA constructs not yielding virus after transfection of Vero cells were used to transfect C6/36 cells as follows. Transfection mixtures, as described above, were added, along with 1 ml of MEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 2 mM non-essential amino acids, and 0.05 mg/ml gentamicin, to monolayers of C6/36 cells. Transfected cell monolayers were incubated at 32°C for 18 hr, cell culture medium was removed and replaced with 2 ml fresh medium, and cell monolayers were incubated at 32°C. After 5 to 6 days, cell culture media were then used to infect Vero cells and incubated for 5-6 days, at which time cell culture media were collected, frozen and titered as described above.

[0141] Recovered viruses were biologically cloned by two rounds of terminal dilution in Vero cells followed by an additional amplification in Vero cells. Briefly, virus was initially diluted to a concentration of approximately 20 PFU/ml in VP-SFM and then subjected to a series of two-fold dilutions across a 96-well plate. Virus dilutions were used to

infect Vero cell monolayers in a 96-well plate and incubated for 5 to 6 days at 35°C. Following incubation, cell culture media were removed and temporarily stored at 4°C, and the virus-positive cell monolayers were identified by immunoperoxidase staining. Terminal dilution was achieved when $\leq$ 25% of cell monolayers were positive for virus. Cell culture medium from a positive monolayer at the terminal dilution was subjected to an additional round of terminal dilution. Following the second terminal dilution, virus was amplified in Vero cells (75 cm^2 flask), collected and frozen as previously described.

[0142] Assays for temperature-sensitivity and mouse attenuation. Assay of the level of temperature sensitivity of the charge-cluster-to-alanine mutant viruses in Vero and HuH-7 cells and their level of replication in the brain of suckling mice were conducted as described in Example 1 and assay of the level of replication in SCID-HuH-7 mice was conducted as described in Example 3.

[0143] Charge-cluster-to-alanine mutant viruses are viable and show temperature-sensitive and mouse attenuation phenotypes. Of 80 full-length DEN4 cDNA constructs containing a single pair of charge-to-alanine mutations, virus was recovered from 32 in either Vero or C6/36 cells (Figure 5). The level of temperature sensitivity of wt rDEN4, rDEN4Δ30, and the 32 mutant viruses is summarized in Table 20. One mutant virus (645-646) was *ts* in Vero but not HuH-7 cells and 7 mutant viruses were *ts* in HuH-7 but not Vero cells. Such mutants whose temperature sensitivity is host-cell dependent are referred to as temperature-sensitive, host-range (*tshr*) mutants. Thirteen mutant viruses were *ts* in both cell types, and 11 mutant viruses were not *ts* on either cell type. Thus a total of 21 mutant viruses were *ts* with 8 mutant viruses exhibiting an *tshr* specificity. None of the mutant viruses showed a small plaque phenotype at permissive temperature. Mutant viruses showed a wide range (0 to 10,000-fold) of restricted replication in suckling mouse brain (Table 20). Fourteen mutant viruses were attenuated in suckling mouse brain, arbitrarily defined as a $\geq$ 1.5 $\log_{10}$–unit reduction in virus titer. There was no correlation between attenuation in mouse brain and temperature sensitivity in either Vero cells (Kendall Rank correlation: $P = 0.77$) or HuH-7 cells (Kendall Rank correlation: $P = 0.06$).

[0144] Thirteen mutant viruses that either showed an *att* phenotype in suckling mouse brain or whose unmutated charged amino acid pair was highly conserved among the

four DEN serotypes (see Example 7) were assayed for *att* in SCID-HuH-7 mice (Table 21). Three of these mutant viruses showed >100-fold decrease in replication relative to wild type DEN4. Overall, mean log reduction from wild type in suckling mice did not show significant correlation with mean log reduction in SCID-HuH-7 mice (Spearman rank correlation, N = 13, P = 0.06). However, mutant virus 200-201 was unusual in that it showed a high level of restriction in SCID-HuH-7 mice but little restriction in suckling mouse brain. When virus 200-201 was removed from the analysis, restriction of replication in suckling and SCID-HuH-7 mice showed a significant correlation (Spearman rank correlation, N = 12, P = 0.02).

[0145] Combining charge-cluster-to-alanine mutations present in two viruses into one virus can enhance its *ts* and *att* phenotypes. Six paired mutations were combined into fourteen double-pair mutant viruses, of which six could be recovered in Vero or C6/36 cells (Table 22). All of the individual paired mutations used in double-pair mutant viruses were *ts* on HuH-7 cells, none was *ts* in Vero cells, and for all combinations at least one mutation pair conferred an *att* phenotype in suckling mouse brain. Evaluation of four of the double-pair mutant viruses (Table 23) revealed that combining charge-cluster-to-alanine mutation pairs invariably resulted in the acquisition of a *ts* phenotype in Vero cells (4 out of 4 viruses) and often resulted in a lowered shutoff temperature in HuH-7 cells (3 out of 4 viruses). In half of the viruses assayed, combination of charge-cluster-to-alanine mutation pairs resulted in enhanced restriction of replication (10-fold greater than either component mutation) in suckling mouse brain (Table 23) and in SCID-HuH-7 mice (Table 24).

[0146] Summary. The major usefulness of the charge-cluster-to-alanine mutations stems from their design: they are located in the DEN4 non-structural gene region and therefore are envisioned as being useful to attenuate DEN4 itself as well as antigenic chimeric viruses possessing the DEN4 NS gene region. Furthermore, they are predicted to be phenotypically more stable than the single-nucleotide substitution mutant viruses such as the 5-FU mutant viruses. Finally, combinations of mutations are envisioned as being created in order to fine-tune attenuation and to further stabilize attenuation phenotypes.

Example 5

Identification and Characterization of DEN4 Mutant Viruses Restricted in Replication in Mosquitoes

SECTION 1. Identification of viruses showing restriction of replication in mosquitoes.

[0147] In Examples 1 and 4, DEN4 mutant viruses were generated through 5-FU mutagenesis and charge-cluster-to-alanine mutagenesis, respectively, in order to identify mutations that confer *ts*, *sp* and *att* phenotypes. Another highly desirable phenotype of a dengue virus vaccine is restricted growth in the mosquito host. A dengue virus vaccine candidate should not be transmissible from humans to mosquitoes in order to prevent both the introduction of a dengue virus into an environment in which it is currently not endemic and to prevent the possible loss of the attenuation phenotype during prolonged replication in an individual mosquito host. Loss of the attenuation phenotype could also occur following sustained transmission between humans and mosquitoes. Recently, loss of attenuation of a live attenuated poliovirus vaccine was seen following sustained transmission among humans (CDC 2000 *MMWR* **49**:1094).

[0148] In the present example, a panel of 1248 DEN4 mutant viruses generated through 5-FU mutagenesis and 32 DEN4 mutant viruses generated through charge-cluster-to-alanine mutagenesis were assayed for restricted growth in mosquito cells. This is a useful preliminary assay for restriction *in vivo*, since restriction in cultured mosquito cells is often, though not always, associated with poor infectivity for mosquitoes (Huang, C.Y. *et al.* 2000 *J Virol* **74**:3020-8). Mutant viruses that showed restriction in mosquito cells and robust growth in Vero cells (the substrate for vaccine development, as discussed in Example 6) were targeted for further characterization.

[0149] **Generation and characterization of the 5-1A1 mutant**. The generation and isolation of the panel of 1248 5-FU mutant viruses and the panel of 32 charge-cluster-to-alanine mutant viruses have been described in Examples 1, 2, and 4. Vero and C6/36 cells were maintained as described in Example 1.

[0150] Each of the 1248 5-FU mutant viruses and 32 charge-cluster-to-alanine mutant viruses was titered in C6/36 cell monolayers in 24-well plates at 32°C and 5% CO_2. After 5 days, plaques were immunostained with anti-DEN4 rabbit polyclonal antibody and

counted as described in the preceding Examples. Mutant viruses were assayed for one of two phenotypes indicating restricted growth in mosquito cells: either *sp* in C6/36 cells relative to Vero cells or a $\geq$ 3.5 $\log_{10}$ PFU/ml decrease in titer between Vero and C6/36 cells at the permissive temperature for each cell type. Two mutant viruses, one generated by 5-FU mutagenesis (#5) and one generated by charge-cluster-to-alanine mutagenesis (rDEN4-356,357), showed reduced plaque size in C6/36 cells. After three terminal dilutions, the 5-FU mutant #5, designated 5-1A1, maintained the reduced plaque size phenotype. Additionally, recombinant virus rDEN4-7546, tested for Vero cell adaptation (discussed in detail in Example 6) also showed reduced plaque size in C6/36 (Figure 10).

[0151] The multicycle growth kinetics of both 5-1A1 and the recombinant wild type rDEN4 in C6/36 cells were determined as described in Example 1. Briefly, cells were infected in triplicate at a multiplicity of infection of 0.01 and samples were harvested at 24-hr intervals. Samples were flash frozen and titered in a single assay in Vero cell monolayers.

[0152] **Oral infection of mosquitoes.** *Aedes aegypti* is one of the primary vectors of dengue virus (Gubler, D.J. 1998 *Clin Microbiol Rev* **11**:480-96). This species was reared at 26°C and 80% relative humidity (RH) with a 16 hr daylight cycle. Adults were allowed continuous access to a cotton pad soaked in a 10% sucrose solution. Five to ten day old female *Ae. aegypti* which had been deprived of a sugar source for 48 hr were fed a bloodmeal consisting of equal volumes of washed human red blood cells, 10% sucrose solution, and dengue virus suspension. The infected blood meal was prepared immediately prior to feeding and offered to mosquitoes in a water-jacketed feeder covered in stretched parafilm and preheated to 38°C (Rutledge, L.C. *et al.* 1964 *Mosquito News* **24**:407-419). Mosquitoes that took a full bloodmeal within 45 min were transferred to a new container by aspirator and maintained as described above. After 21 days, mosquitoes were stored at -20°C until dissection.

[0153] **Intrathoracic inoculation of mosquitoes.** The large, non-haematophagous mosquito *Toxorhynchites splendens* is a sensitive host for determining the infectivity of dengue virus. This species was reared at 24°C and 75% RH with a 12 hr daylight cycle. Larvae and pupae were fed on appropriately sized *Aedes* larvae; adults were allowed continuous access to a cotton pad soaked in a 10% sucrose solution. Groups of one

to ten day old adult *T. splendens* of both sexes were immobilized by immersion of their container in an icewater bath and inoculated intrathoracically with undiluted virus and serial tenfold dilutions of virus in 1X PBS. Virus was inoculated in a 0.22 µl dose using a Harvard Apparatus microinjector (Medical Systems Corp, Greenvale NY) and a calibrated glass needle (technique is a modification of the method described in Rosen and Gubler, 1974).

[0154] Detection of viral antigen in body and head tissues by immunofluorescence assay (IFA). Head and midgut preparations of *Aedes aegypti* and head preparations of *Toxorhynchites splendens* were made on glass slides as described in Sumanochitrapon *et al.* (Sumanochitrapon, W. *et al.* 1998 *Am J Trop Med Hyg* **58**:283-6). Slides were fixed in acetone for 20 min, and placed at 4°C until processed by IFA. The primary antibody, hyperimmune mouse ascites fluid specific for DEN-4 (HMAF), was diluted 1/100 in PBS-Tween 20 (0.05%). Slides were incubated at 37°C in a humid chamber for 30 min, and subsequently rinsed in PBS-Tween 20. The secondary antibody, FITC conjugated goat anti-mouse IgG (KPL, Gaithersburg, MD), was diluted 1/200 in PBS-Tween 20 with 0.002% Evan's Blue. Slides were viewed on an Olympus BX60 microscope. The infectious dose required to infect 50% of mosquitoes (ID_{50}) was determined by the method of Reed and Muench (Reed, L.J. & Muench, H. 1938 *Am J Hyg* 27:493-497). For *Aedes aegypti* infections, two OID_{50} (oral infectious dose 50) values were calculated for each virus: the OID_{50} required to produce an infection in the midgut, with or without dissemination to the head, and the OID_{50} required to produce disseminated infection. For *Tx. splendens* one MID_{50} (mosquito infectious dose 50) value was calculated.

[0155] Statistical Analysis. The percentage of mosquitoes infected by different viruses were compared using logistic regression analysis (Statview, Abacus Inc.).

[0156] Mutations restricting growth of DEN4 in mosquito cells but not Vero cells are rare. Out of 1280 mutant viruses initially assayed, only two, #5 and rDEN4-356,357, showed reduced plaque size in C6/36 cells and normal plaque size in Vero cells. One additional virus, rDEN4-7546 (described in Example 6), with reduced plaque size in C6/36 was detected in subsequent assays. Mutant virus #5 was cloned by three successive terminal dilutions and designated 5-1A1; rDEN4-7546 and rDEN4-356,357 had already been twice-terminally diluted when they were tested in C6/36 cells. Virus 5-1A1 has been

extensively characterized and its phenotypes are described in detail in the following section. rDEN4-356,357 and rDEN4-7546 are envisioned as being characterized in a similar fashion.

[0157] Plaque size and growth kinetics of 5-1A1. 5-1A1 replicated to 6.7 log_{10}PFU/ml in Vero cells with normal plaque size and replicated to 7.6 log_{10}PFU/ml in C6/36 cells with small plaque size (Figure 6, Table 25). In comparison, wild type DEN4 used as a concurrent control replicated to 7.3 log_{10}PFU/ml in Vero cells, 8.3 log_{10}PFU/ml in C6/36 cells, and showed normal plaque size in both cell types (Figure 6, Table 25). The growth kinetics of 5-1A1 was compared to that of wild type DEN4 by infecting C6/36 cells at an MOI of 0.01 and monitoring the production of infectious virus. The kinetics and magnitude of replication of 5-1A1 in C6/36 cells was comparable to that of wild type DEN4 (Figure 7).

[0158] 5-1A1 is restricted in its ability to infect mosquitoes. 5-1A1 was evaluated for its ability to infect *Aedes aegypti* mosquitoes through an artificial bloodmeal (Table 26). In this assay the ability to infect the midgut of the mosquito and the ability for a midgut infection to disseminate to the head are measured separately. The oral infectious dose 50 (OID_{50}) of wild type DEN4 for the midgut was 3.3 log_{10} PFU; the OID_{50} of wild type DEN4 for a disseminated infection was 3.9 log_{10} PFU. In contrast, 5-1A1 never infected 50% of mosquitoes at the doses used. In order to calculate the OID_{50} for midgut infections by 5-1A1, it was assumed that at a 10-fold higher dose, 100% of 25 mosquitoes would have become infected. Using this assumption, the conservative estimate of the OID_{50} for midgut infections by 5-1A1 was $\geq$ 3.9 log_{10}PFU. Because 5-1A1 produced only 3 disseminated infections, we did not attempt to calculate an OID_{50} for this category. 5-1A1 was significantly restricted in its ability to infect the midgut relative to wild type DEN4 (logistic regression, N = 150, $P < 0.001$). Additionally, 5-1A1 produced very few disseminated infections, but because of low numbers this result was not amenable to statistical analysis.

[0159] 5-1A1 was also significantly restricted in its ability to infect *Tx. splendens* mosquitoes following intrathoracic inoculation (Table 27). The MID_{50} of wild type DEN4 was 2.3 log_{10} PFU whereas the MID_{50} of 5-1A1 was estimated to be > 3.0 log_{10} PFU (logistic regression, N = 36, $P < 0.01$).

[0160] **5-1A1 does not show a *ts* or an *att* phenotype.** 5-1A1 was tested for temperature sensitivity in Vero and HuH-7 cells and for attenuation in suckling mouse brains as described in Example 1. The mutant virus was not temperature sensitive, as defined in Example 1, and was not attenuated in suckling mouse brain (Table 25).

[0161] **Identification and confirmation of the mutation responsible for the phenotype of 5-1A1.** The nucleotide sequence of the entire genome of 5-1A1 was determined as described in Example 1. Sequencing of 5-1A1 revealed three changes from the wild type sequence: two translationally-silent point mutations at positions 7359 and 9047, and one coding point mutation (C to U) at position 7129 in the NS4B gene which resulted in a proline to leucine substitution.

[0162] To formally confirm the effect of the C7129U mutation, the mutation was inserted into the cDNA p4, which has been described in Examples 1, 2, and 3 and in Figure 4, using Kunkel mutagenesis as described in Examples 1 and 3. The mutagenized cDNA was transcribed and transfected as described in Example 3, and the resulting virus, after two terminal dilutions, was designated rDEN4-7129-1A. Like 5-1A1, rDEN4-7129-1A showed normal plaque size and titer in Vero cells and reduced plaque size and normal titer in C6/36 cells (Table 25). rDEN4-7129-1A was not *ts* on either Vero or HuH-7 cells and was not *att* in suckling mouse brain. Additionally, rDEN4-7129-1A did not show the SCID-HuH-7 *att* phenotype described in Example 3 (Table 25). The ability of rDEN4-7129-1A to infect mosquitoes is envisioned as being tested in both *Ae. aegypti* and *Tx. splendens*.

[0163] To test the compatibility of the C7129U mutation and the Δ30 deletion, the C7129U mutation was inserted into rDEN4Δ30 using previously described techniques. The resulting virus, designated rDEN4Δ30-7129, is envisioned as being tested for the phenotypes listed in Table 25.

[0164] In summary, three mutant viruses, 5-1A1, rDEN4-356,357 and rDEN4-7546, showed a particular combination of phenotypes characterized by normal plaque size and replication to high titers in Vero cells and small plaque size but unrestricted growth in mosquito cells. 5-1A1 was further characterized and lacked temperature sensitivity in either Vero or HuH-7 cells and showed normal levels of replication in mouse brain and in SCID-HuH-7 mice and restricted infectivity for both *Ae. aegypti* and *Tx. splendens* mosquitoes. In

comparison to wild type rDEN4, the 5-1A1 mutant had one coding mutation: a point mutation (C to U) at nucleotide 7129 in NS4B resulting in a replacement of Pro with Leu. Because 5-1A1 contains only a single missense mutation, the phenotype of this mutant virus can be attributed to the effect of the mutation at position 7129. These results indicate that the 7129 mutation is responsible for the phenotype of decreased infectivity for mosquitoes and is predicted to be useful to restrict replication of vaccine candidates in mosquitoes. To formally confirm this, we have inserted the 7129 mutation into a recombinant DEN4 virus. The resulting virus, designated rDEN4-7129-1A, shows an absence of *ts* and *att* phenotypes similar to 5-1A1. It is envisioned as being tested for mosquito infectivity.

[0165] The 7129 mutation is a valuable point mutation to include in a DEN4 vaccine candidate and into each of the dengue virus antigenic chimeric vaccine candidates since its biological activity is host specific, i.e., it is restricted in replication in mosquitoes but not in mammals. Moreover, as discussed in Example 6, the 7129 mutation has also been shown to enhance replication in Vero cells. Thus, its insertion into a vaccine candidate is envisioned as enhancing vaccine production in tissue culture without affecting the biological properties specified by other attenuating mutations. It is also envisioned as providing a useful safeguard against mosquito transmission of a dengue virus vaccine.

SECTION II. Design of mutations to restrict replication in mosquitoes

[0166] In Section 1 of Example 5, we screened a large panel of mutant viruses carrying both random mutations (generated with 5-fluorouracil) and specific mutations (generated through charge-cluster-to-alanine mutagenesis) for restricted growth in C6/36 cells, a proxy measure for restriction in mosquitoes. However, in neither case were mutations designed for the specific purpose of restricting replication in mosquitoes. In this section, we identified nucleotide sequences in the 3' UTR that show conserved differences between the mosquito-transmitted and tick-transmitted flaviviruses. We then altered those sequences in the DEN4 cDNA p4 by either deleting them altogether or exchanging them with the homologous sequence of the tick-transmitted Langat virus. The resulting viruses were assayed for reduced plaque size and titer in both Vero and C6/36 cells and for infectivity for *Ae. aegypti* and *Tx. splendens*.

[0167] **Identification and modification of particular 3' UTR sequences showing conserved differences between vectors.** Several studies (Olsthoorn, R.C. & Bol, J.F. 2001 *RNA* 7:1370-7; Proutski, V. *et al.* 1997 *Nucleic Acids Res* **25**:1194-202) have identified conserved differences in the nucleotide sequences of the 3' UTR of mosquito-transmitted and tick-transmitted flaviviruses. Such differences are concentrated in the 3' terminal core region, the approximately 400 3' terminal nucleotides. It has been suggested that these sequences may have a vector-specific function (Proutski, V. *et al.* 1997 *Nucleic Acids Res* **25**:1194-202). While such a function has not been identified, it may nonetheless be possible to disrupt vector infectivity by deleting or otherwise altering these nucleotides.

[0168] To identify target sequences for this type of alteration, we constructed an alignment of the 3' UTR nucleotide sequences of seven mosquito-transmitted flaviviruses and four tick-transmitted flaviviruses (Figure 8). From this alignment, we identified several sequences that showed conserved differences between the mosquito-transmitted flaviviruses and tick-transmitted flaviviruses. We then designed primers to alter these sequences in the wt DEN4 cDNA p4 (Figure 4) in one of two ways: 1) deletion of the nucleotides (Δ) or 2) replacement of the nucleotides with the homologous sequence from the tick-transmitted flavivirus Langat (swap). Langat was chosen as the template for swapped nucleotides because it is naturally attenuated (Pletnev, A.G. 2001 *Virology* **282**:288-300), and therefore unlikely to enhance the virulence of rDEN4 virus derived from the modified cDNA. The DEN4 sequences altered and the mutagenesis primers used to do so are listed in Table 28. Nucleotides 10508-10530 correspond to the CS2 region identified in previous studies (Proutski, V. *et al.* 1997 *Nucleic Acids Res* **25**:1194-202).

[0169] Mutagenesis of p4, transcription and transfection were conducted as previously described in Section I of this Example. All five of the engineered viruses were recovered, and all were subjected to two rounds of terminal dilution as previously described.

[0170] **Evaluation of phenotypes: cell culture.** Viruses were titered in Vero and C6/36 cells as previously described, and the results are listed in Table 29. All of the viruses replicated to >5.0 $\log_{10}$ PFU/ml; one of them (rDEN4Δ10508-10530) replicated to > 8.0 $\log_{10}$ PFU/ml. Only one of the viruses (rDEN4Δ10535-10544) was small plaque in C6/36 cells; this virus showed wild-type plaque size in Vero cells. Interestingly, another virus

(rDEN4swap10508-10539) showed wild type plaque size in C6/36 cells but was *sp* in Vero cells.

[0171] Evaluation of phenotypes: mosquito infectivity. To date one of the five viruses has been tested for infectivity via intrathoracic inoculation in *Tx. splendens*, using previously described methods. Virus rDEN4Δ10508-10530 was dramatically restricted in infectivity relative to the wild type (Table 30). So few mosquitoes were infected that it was not possible to calculate an MID_{50} for this virus.

[0172] One of the five viruses has been tested for infectivity of *Ae. aegypti* fed on an infectious bloodmeal using previously described methods. rDEN4swap10535-10544 (Table 31) caused significantly fewer midgut infections than wild type rDEN4, but the percentage of disseminated infections did not differ between rDEN4swap10535-10544 and wild type rDEN4. All of the viruses are envisioned as being tested for mosquito infectivity using both methods.

[0173] Summary. In this example we have outlined two different strategies for preventing mosquito transmission of a dengue vaccine. First, several small substitution mutations, including two point mutations and one paired charge-to-alanine substitution, have been shown to restrict the replication of DEN4 in mosquito C6/36 cells in cell culture, and one of these mutations (C7129U) has been shown to restrict the ability of DEN4 virus to infect mosquitoes. Second, we have created a variety of deletion and substitution mutations in regions of the DEN4 3' UTR that show conserved differences between mosquito-transmitted and tick-transmitted flaviviruses. One of these viruses is *sp* in C6/36 cells and at least two of these viruses show some degree of restriction of mosquito infectivity. By design, the nucleotide sequences in which these mutations were made are highly conserved among the four dengue serotypes and among mosquito-transmitted flaviviruses in general, indicating that they are portable to other vaccine candidates for mosquito-borne flaviviruses. All of the mutations discussed in this Example lie outside the structural genes and so are envisioned as being useful in constructing antigenic-chimeric vaccine candidates.

Example 6

Adaptation Mutations Which Enhance the Replication of DEN4 and DEN4 Chimeric Viruses in Vero Cells.

[0174] Vero cells are a highly characterized substrate that should be suitable for the manufacture of live attenuated flavivirus vaccines, such as dengue virus and tick-borne encephalitis virus. In addition, Vero cells can also be used to grow flaviviruses to high titer for the preparation of an inactivated virus vaccine. Optimal sequences for the efficient growth of dengue viruses in Vero cells have not been identified, but it is well known that flaviviruses accumulate mutations during passage in various cell cultures (Dunster, L.M. *et al.* 1999 *Virology* **261**:309-18; Theiler, M. & Smith, H.H. 1937 *J Exp Med* **65**:787-800). Inclusion of specific sequences in live attenuated viruses that enhance their replication in Vero cells and increase the number of doses of vaccine produced per unit substrate would greatly facilitate their manufacture. Similarly, inclusion of Vero cell growth-promoting sequences in wild type viruses used for the preparation of an inactivated virus vaccine would also greatly facilitate the manufacture of the vaccine. The present example identifies mutations that occur following passage of DEN4 virus and DEN2/4 chimeric viruses in Vero cells. Data derived from five sources provided information for this analysis making it possible to generate a list of Vero cell growth-promoting sequences.

[0175] **Presence of identical mutations in multiple 5-FU mutant viruses.** First, as described in Examples 1 and 2, the genomes of 42 dengue virus clones isolated from a 5-FU mutagenized stock of virus were completely sequenced. If mutations that enhance replication occurred during the passage of these 42 mutant viruses in Vero cells, then such mutations should reveal themselves by representation in more than one clone. Analysis of the 42 sequences revealed the occurrence of specific missense mutations in coding regions or nucleotide substitutions in UTRs in multiple clones that are not present in the 2A parental virus genome (Tables 11 and 32). These mutations, many of which occur within a 400 nucleotide section of the NS4B coding region, represent Vero cell-adaptation mutations. One mutation, such as the 4995 mutation, present in eight viruses was found to specify both *ts* and *att* phenotypes (Examples 1 and 3). In contrast, the 7163 mutation, present in six viruses,

does not specify a *ts* or *att* phenotype (Table 13) and thus is an example of a specific Vero cell growth-promoting mutation.

[0176] Presence of Vero cell adaptation mutations in other DEN4 viruses and DEN2/4 antigenic chimeric viruses. Second, the 2A-13 dengue virus that was used as a parallel passaged wild type control during the 5-FU experiments described in Example 1 was grown and cloned in Vero cells in the absence of 5-FU in a manner identical to that of the 5-FU treated viruses. Sequence analysis of this 5-FU untreated virus, designated 2A-13-1A1, revealed that the virus genome contained a mutation at nucleotide 7163 (Example 1 and Table 32), identical to the missense mutation previously identified in 6 of the 5-FU mutant viruses (Tables 11 and 32). This indicates that growth and passage of DEN4 virus in Vero cells is sufficient to acquire this specific mutation, i.e. mutagenesis with 5-FU is not required. Thus, information from two separate sources indicates that the 7163 mutation appeared in separate Vero cell passaged viruses, thereby strengthening the interpretation that this mutation is growth promoting.

[0177] Third, following passage of the 2AΔ30 and rDEN4Δ30 in Vero cells, sequence analysis revealed the presence of a mutation at nucleotides 7153 and 7163, respectively. These two mutations were also previously identified among the 5-FU treated viruses (Table 32). Again, identical mutations appeared following independent passage of virus in Vero cells, corroborating the hypothesis that these mutations confer a growth advantage in Vero cells.

[0178] Fourth, an antigenic chimeric dengue virus vaccine candidate was generated that expressed the structural proteins C, prM, and E from DEN2 on a DEN4 wild type genetic background or an attenuated Δ30 genetic background. To construct this virus, the C, prM and E region of wild type cDNA plasmid p4 was replaced with a similar region from DEN2 virus strain NGC (Figure 10). Specifically, nucleotides between restriction sites *Bgl*II (nt 88) and *Xho*I (nt 2345) of p4 were replaced with those derived from dengue type 2 virus. RNA transcripts synthesized from the resulting p4-D2 plasmid were transfected into Vero cells and rDEN2/4 virus was recovered. A further attenuated version of this chimeric virus containing the Δ30 mutation, rDEN2/4Δ30, was recovered in C6/36 mosquito cells following transfection of cells with RNA transcripts derived p4Δ30-D2. However,

rDEN2/4Δ30 could not be recovered directly in Vero cells. The rDEN2/4Δ30 mutant virus recovered in C6/36 cells replicated to very low levels in Vero cells (<1.0 $\log_{10}$PFU/ml) but grew to high titer in C6/36 cells (>6.0 $\log_{10}$PFU/ml). Genomic sequence of the C6/36-derived virus matched the predicted cDNA sequence and is shown in Appendix 3. Nevertheless, when C6/36-derived rDEN2/4Δ30 was serially passaged 3 to 4 times in Vero cells, a virus population adapted for growth in Vero cells emerged. Virus from this Vero cell-adapted preparation was cloned and amplified in Vero cells to a titer >6.0 $\log_{10}$PFU/ml. The genomic sequence was determined for 2 independent virus clones and compared to the predicted cDNA sequence (Table 33 and 34). Each cloned virus contains a mutation in a non-structural gene which coincides closely in location or sequence with a mutation previously identified among the panel of 5-FU mutagenized viruses. The other mutations in these two clones also might confer a growth advantage in Vero cells. Importantly, the mutations identified in Tables 33 and 34 are absolutely required for replication in Vero cells, and it would not be possible to produce the rDEN2/4Δ30 vaccine candidate in Vero cells without the growth-promoting mutations identified in Tables 33 and 34.

[0179] Fifth, sequence analysis of the dengue 4 wild-type virus strain 814669 (GenBank accession no. AF326573) following passage in Vero cells identified a mutation in the NS5 region at nucleotide 7630 which had previously been identified among the panel of 5-FU mutagenized viruses (Table 32). This mutation at nucleotide 7630 was introduced into recombinant virus rDEN4 by site-directed mutagenesis as described in Table 16. The resulting virus, rDEN4-7630, was not temperature sensitive when tested at 39°C, indicating that mutation 7630 does not contribute to temperature sensitivity.

[0180] **Characterization of rDEN2/4Δ30 chimeric viruses containing single and multiple Vero cell adaptation mutations.** The generation of chimeric virus rDEN2/4Δ30 provided a unique opportunity for evaluating the capacity of individual mutations to promote increased growth in Vero cells. Because rDEN2/4Δ30 replicates to very low titer in Vero cells, yet can be efficiently generated in C6/36 mosquito cells, recombinant virus bearing putative Vero-cell adapting mutations were first generated in C6/36 cells and then virus titers were determined in both C6/36 and Vero cells. As shown in Table 35, addition of a single mutation to rDEN2/4Δ30 resulted in a greater than 1000-fold

increase in titer in Vero cells, confirming the Vero cell adaptation phenotype conferred by these mutations. However, the combination of two separate mutations into a single virus did not increase the titer in Vero cells beyond the level observed for viruses bearing a single adaptation mutation. Inclusion of either the 7182 or 7630 mutation in the cDNA of rDEN2/4Δ30 allowed the virus to be recovered directly in Vero cells, circumventing the need to recover the virus in C6/36 cells.

[0181] Characterization of the growth properties of rDEN4 viruses containing single and multiple defined Vero cell adaptation mutations. To confirm the ability of Vero cell adaptation mutations to enhance growth of DEN4 viruses, site-directed mutagenesis was used to generate rDEN4 viruses encoding selected individual mutations as described in Examples 1 and 3. Five mutations in NS4B (7153, 7162, 7163, 7182, and 7546) from the list of repeated mutations in the 5-FU mutant viruses (Table 32) were introduced singly into the p4 cDNA clone. In addition, the mosquito-restricted, rDEN4-7129 virus was evaluated for enhanced growth in Vero cells since the location of this mutation is in the same region of NS4B. Each virus, including wild-type rDEN4, was recovered, terminally diluted, and propagated in C6/36 cells to prevent introduction of additional Vero cell adaptation mutations, however, because of its restricted growth in C6/36 cells, rDEN4-7129 was propagated only in Vero cells.

[0182] Plaque size was evaluated for each mutant rDEN4 virus in Vero cells and C6/36 cells and compared to wild-type rDEN4. Six-well plates of each cell were inoculated with dilutions of virus and plaques were visualized five days later. Representative plaques are illustrated in Figure 10 and demonstrate that the presence of a Vero cell adaptation mutation does indeed confer increased virus cell to cell spread and growth specifically in Vero cells. In C6/36 cells, average plaque size was approximately 0.50 mm for both wild-type rDEN4 and each mutant virus (except for rDEN4-7546 and rDEN4-7129 which were smaller than wild-type; see Example 5). However, rDEN4 viruses expressing mutation 7162, 7163, 7182, and 7129 had a greater than two-fold increase in plaque size in Vero cells compared to wild-type rDEN4 virus. A smaller but consistent increase in plaque size was observed for rDEN4-7153 and rDEN4-7546.

[0183] Growth kinetics and virus yield in Vero cells was assessed for the same panel of rDEN4 viruses. Vero cells were infected at an MOI of 0.01 and samples were removed daily for 10 days, titered on Vero cells, and plaques were visualized. The results in Figure 11 indicate that the presence of a Vero cell adaptation mutation increased the kinetics of virus growth, but had only a marginal effect on the peak virus yield. At day four post-infection, wild-type rDEN4 grew to 5.2 $\log_{10}$PFU/ml while the level of replication in rDEN4-7129-infected cells was 100-fold higher. The rest of the mutant rDEN4 viruses had an increased yield at day four ranging from 0.9 (rDEN4-7153) to 1.6 (rDEN4-7162 and –7163) $\log_{10}$PFU/ml. Interestingly, enhanced kinetics of virus growth correlated with increased plaque size in Vero cells. The peak virus yield was reached by day 6 post-infection for rDEN4-7129, -7162, -7163, and –7182 while wild-type rDEN4 did not reach peak titer until day 10. However, the peak virus yield was only slightly higher in rDEN4 viruses expressing Vero cell adaptation mutations.

[0184] In an effort to further enhance rDEN4 replication, especially the peak virus yield, combinations of selected Vero cell adaptation mutations were introduced into the rDEN4 background. Three viruses with dual mutations were generated: rDEN4-7153-7163, rDEN4-7153-7182, and rDEN4-7546-7630 and tested in a Vero cell time course infection as described above along with rDEN4 and rDEN4-7162 as a positive control (Figure 12). The viruses expressing combined mutations grew in a nearly identical manner to rDEN4-7162 indicating that these selected combinations did not enhance the kinetics or peak virus yield. Additional combinations of these and other Vero cell adaptation mutations are envisioned as increasing peak virus yield.

[0185] **Discussion.** Some of the growth promoting mutations listed in Table 32 are also found in homologous regions of DEN1, DEN2, and DEN3 and are envisioned as serving to promote the replication of these viruses in Vero cells. Specifically, the growth promoting mutations indicated in Table 32 that are present in a DEN4 virus are envisioned as being useful for importation into homologous regions of other flaviviruses, such as DEN1, DEN2 and DEN3. Examples of such conserved regions are shown in Appendix 4 and are listed in Table 36. The nucleotides for both mutation 7129 and 7182 are conserved in all four dengue virus serotypes. It is also interesting to note that mutation 7129 not only increases

growth in Vero cells (Figure 10), but it also forms small plaques in mosquito cells (Figure 6, Table 25). Lee *et al.* previously passaged DEN3 virus in Vero cells and performed limited sequence analysis of only the structural gene regions of the resulting viruses (Lee, E. *et al.* 1997 *Virology* **232**:281-90). From this analysis a menu of Vero adaptation mutations was assembled. Although none of these mutations correspond to the Vero adaptation mutations identified in this Example, a single mutation at amino acid position 202 in DEN3 corresponds to mutation 1542 identified in 5-FU mutant virus #1012. The current Example emphasizes the importance in this type of study of determining the sequence of the entire viral genome.

[0186] Vero cell growth optimized viruses are envisioned as having usefulness in the following areas. First, the yield of a live attenuated vaccine virus in Vero cells is predicted to be augmented. The live attenuated vaccine candidate is conveniently a DEN4 or other dengue virus or a DEN1/4, DEN2/4, or DEN3/4 antigenic chimeric virus, or a chimeric virus of another flavivirus based on the DEN4 background. The increased yield of vaccine virus is envisioned as decreasing the cost of vaccine manufacture. Second, Vero cell adaptation mutations that are attenuating mutations, such as the 4995 mutation, are envisioned as being stable during the multiple passage and amplification of virus in Vero cell cultures that is required for production of a large number of vaccine doses. Third, Vero cell adaptation mutations are actually required for the growth of the rDEN2/4Δ30 vaccine candidate in Vero cells. Fourth, the increase in yield of a DEN wild type or an attenuated virus is envisioned as making it economically feasible to manufacture an inactivated virus vaccine: Fifth, the presence of the Vero cell growth promoting mutations in the DEN4 vector of the rDEN1/4, rDEN2/4, and rDEN3/4 antigenic chimeric viruses or other flavivirus chimeric viruses based on DEN4 is envisioned as permitting the viruses to grow to a high titer and as thereby being useful in the manufacture of a inactivated virus vaccine. Sixth, the insertion of Vero cell growth promoting mutations into cDNAs such as rDEN2/4Δ30 is envisioned as permitting recovery of virus directly in Vero cells, for which there are qualified master cell banks for manufacture, rather than in C6/36 cells for which qualified cell banks are not available. And seventh, insertion of the 7129 and 7182 mutations into DEN1, DEN2,

or DEN3 wt viruses is envisioned as increasing their ability to replicate efficiently and be recovered from cDNA in Vero cells.

Example 7

Assembly of a List of Attenuating Mutations

[0187] The data presented in these examples permits the assembly of a list of attenuating mutations that is summarized in Table 37. This list contains individual mutations identified in Tables 13 – 16, 20, and 21 that are known to independently specify an attenuation phenotype. Mutation 7129 is also included since it is derived from virus 5-1A1 shown to be attenuated in mosquitoes. We envision using various combinations of mutations from this list to generate viruses with sets of desirable properties such as restricted growth in the liver or in the brain as taught in Example 3 (Table 18) and Example 4 (Tables 23 and 24). These mutations are also combinable with other previously described attenuating mutations such as the Δ30 mutation, as taught in Example 1 (Table 6) and Example 3 (Table 19) to produce recombinant viruses that are satisfactorily attenuated and immunogenic. Mutations listed in Table 37 are also envisioned as being combined with other previously described attenuating mutations such as other deletion mutations or other point mutations (Blok, J. *et al.* 1992 *Virology* **187**:573-90; Butrapet, S. *et al.* 2000 *J Virol* **74**:3011-9; Men, R. *et al.* 1996 *J Virol* **70**:3930-7; Puri, B. *et al.* 1997 *J Gen Virol* **78**:2287-91).

[0188] The possibility of importing an attenuating mutation present in one paramyxovirus into a homologous region of a second paramyxovirus has recently been described (Durbin, A.P. *et al.* 1999 *Virology* **261**:319-30; Skiadopoulos, M.H. *et al.* 1999 *Virology* **260**:125-35). Such an importation confers an *att* phenotype to the second virus or, alternatively, further attenuates the virus for growth *in vivo*. Similarly we envision importing an attenuating mutation present in one flavivirus to a homologous region of a second flavivirus which would confer an *att* phenotype to the second flavivirus or, alternatively, would further attenuate the virus for growth *in vivo*. Specifically, the attenuating mutations indicated in Table 37 are envisioned as being useful for importation into homologous regions of other flaviviruses. Examples of such homologous regions are indicated in Appendix 4 for the mutations listed in Table 37.

Example 8

Evaluation of Dengue Virus Vaccine In Humans And Rhesus Monkeys

[0189] The present example evaluates the attenuation for humans and rhesus monkeys (as an animal model) of a DEN-4 mutant bearing a 30 nucleotide deletion (Δ30) that was introduced into its 3' untranslated region by site-directed mutagenesis and that was found previously to be attenuated for rhesus monkeys (Men, R. *et al.* 1996 *J Virol* **70**:3930-7), as representative of the evaluation of any dengue virus vaccine for attenuation in humans and rhesus monkeys (as an animal model).

[0190] **Viruses and cells.** The wild type (wt) DEN-4 virus strain 814669 (Dominica, 1981), originally isolated in *Aedes pseudoscutellaris* (AP61) cells, was previously plaque-purified in LLC-MK2 cells and amplified in C6/36 cells as described (Mackow, E. *et al.* 1987 *Virology* **159**:217-28). For further amplification, the C6/36 suspension was passaged 2 times in Vero (WHO) cells maintained in MEM-E (Life Technologies, Grand Island, NY) supplemented with 10% FBS. Viruses derived from RNA transfection or used for clinical lot development were grown in Vero (WHO) cells maintained in serum-free media, VP-SFM (Life Technologies).

[0191] **Construction of DEN-4 deletion mutants.** A 30 nucleotide (nt) deletion was previously introduced into the 3′ untranslated region of the 2A cDNA clone of wt DEN-4 strain 814669 as described (Men, R. *et al.* 1996 *J Virol* **70**:3930-7). This deletion removes nucleotides 10478 - 10507, and was originally designated 3′d 172-143, signifying the location of the deletion relative to the 3′ end of the viral genome. In the current example, this deletion is referred to as Δ30. The full-length 2A cDNA clone has undergone several subsequent modifications to improve its ability to be genetically manipulated. As previously described, a translationally-silent *Xho*I restriction enzyme site was engineered near the end of the E region at nucleotide 2348 to create clone 2A-*Xho*I (Bray, M. & Lai, C.J. 1991 *PNAS USA* **88**:10342-6). In this example, the viral coding sequence of the 2A-*Xho*I cDNA clone was further modified using site-directed mutagenesis to create clone p4: a unique *Bbv*CI restriction site was introduced near the C-prM junction (nucleotides 447 - 452); an extra *Xba*I restriction site was ablated by mutation of nucleotide 7730; and a unique *Sac*II restriction site was created in the NS5 region (nucleotides 9318 - 9320). Each of these engineered mutations

is translationally silent and does not change the amino acid sequence of the viral polypeptide. Also, several mutations were made in the vector region of clone p4 to introduce or ablate additional restriction sites. The cDNA clone p4Δ30 was generated by introducing the Δ30 mutation into clone p4. This was accomplished by replacing the *Mlu*I - *Kpn*I fragment of p4 (nucleotides 10403 - 10654) with that derived from plasmid 2AΔ30 containing the 30 nucleotide deletion. The cDNA clones p4 and p4Δ30 were subsequently used to generate recombinant viruses rDEN4 and rDEN4Δ30, respectively.

[0192] Generation of viruses. Full-length RNA transcripts were synthesized from cDNA clones 2A and 2AΔ30 using SP6 RNA polymerase as previously described (Lai, C.J. *et al.* 1991 *PNAS USA* **88**:5139-43; Men, R. *et al.* 1996 *J Virol* **70**:3930-7). The reaction to generate full-length RNA transcripts from cDNA clones p4 and p4Δ30 was modified and consisted of a 50 µl reaction mixture containing 1 µg linearized plasmid, 60 U SP6 polymerase (New England Biolabs (NEB), Beverly, MA), 1X RNA polymerase buffer (40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol), 0.5 mM m7G(5′)ppp(5′)G cap analog (NEB), 1 mM each nucleotide triphosphate, 1 U pyrophosphatase (NEB), and 80 U RNAse inhibitor (Roche, Indianapolis, IN). This reaction mixture was incubated at 40°C for 90 min and the resulting transcripts were purified using RNeasy mini kit (Qiagen, Valencia, CA). For transfection of Vero cells, purified transcripts (1 µg) were mixed with 12 µl DOTAP liposome reagent (Roche) in saline containing 20 mM HEPES buffer (pH 7.6) and added to cell monolayer cultures in a 6-well plate. After 5 - 17 days, tissue culture medium was harvested, clarified by centrifugation, and virus was amplified in Vero cells. The presence of virus was confirmed by plaque titration. It should be noted that during the course of transfection and amplification of 2AΔ30 to create the vaccine lot, the virus underwent a total of 6 passages entirely in Vero cells. The remaining viruses, rDEN4 and rDEN4Δ30 were passaged 5 times in Vero cells to generate the virus suspension used for sequence analysis and studies in rhesus monkeys.

[0193] Vaccine Production. An aliquot of clarified tissue culture fluid containing vaccine candidate 2AΔ30 was submitted to DynCorp (Rockville, MD) for amplification of virus in Vero cells and production of a vaccine lot. For vaccine production, 2AΔ30 infected tissue culture supernatant was harvested, SPG buffer added (final

concentration: 218 mM sucrose, 6 mM L-glutamic acid, 3.8 mM potassium phosphate, monobasic, and 7.2 mM potassium phosphate, dibasic, pH 7.2), and the virus suspension was clarified by low speed centrifugation. To degrade residual Vero cell DNA, the vaccine suspension was treated with Benzonase endonuclease (American International Chemical, Natick, MA), 100 U/ml and incubated for 1 hr at 37°C, followed by high-speed centrifugation (17,000 x g, 16 hr). The resulting virus pellet was gently rinsed with MEM-E, resuspended in MEM-E containing SPG, sonicated, distributed into heat-sealed ampoules, and stored frozen at -70°C. Final container safety testing confirmed microbial sterility, tissue culture purity, and animal safety. The 2AΔ30 vaccine lot (designated DEN4-9) has a titer of 7.48 log10PFU/ml, with a single dose of 5.0 log10 PFU/ml containing <1 pg/ml Vero cell DNA and <0.001 U/ml Benzonase endonuclease.

[0194] Sequence of cDNA clones and viral genomes. The nucleotide sequence of the viral genome region of cDNA plasmids 2A and p4 was determined on a 310 genetic analyzer (Applied Biosystems, Foster City, CA) using vector-specific and DEN-4-specific primers in BigDye terminator cycle sequencing reactions (Applied Biosystems). The nucleotide sequence of the genomes of the parental wt DEN-4 strain 814669 and of recombinant viruses 2A wt, 2AΔ30 (vaccine lot), rDEN4, and rDEN4Δ30 was also determined. Viral RNA was extracted from virus preparations and serum samples using the QIAamp Viral RNA mini kit (Qiagen). Reverse transcription (RT) was performed using random hexamers and the SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies). Overlapping PCR fragments of approximately 2000 base pairs were generated using optimized DEN-4 specific primers and Advantage cDNA polymerase (ClonTech, Palo Alto, CA). Both strands of purified PCR fragments were sequenced directly using dye-terminator reactions as described above and results were assembled into a consensus sequence. To determine the nucleotide sequence of the viral RNA 5′ and 3′ regions, the 5′ cap nucleoside of the viral RNA was removed with tobacco acid pyrophosphatase (Epicentre, Madison, WI) followed by circularization of the RNA using RNA ligase (Epicentre). RT-PCR was performed as described and a cDNA fragment spanning the ligation junction was sequenced using DEN-4 specific primers. GenBank

accession numbers have been assigned as follows (virus: accession number): 814669: AF326573, 2AΔ30: AF326826, rDEN4: AF326825, and rDEN4Δ30: AF326827.

[0195] Human Vaccine Recipients. 20 normal healthy adult volunteers were recruited by the Johns Hopkins School of Hygiene and Public Health Center for Immunization Research (CIR) located in Baltimore, Maryland. The clinical protocol was reviewed and approved by the Joint Committee for Clinical Investigation of the Johns Hopkins University School of Medicine and informed consent was obtained from each volunteer. Volunteers were enrolled in the study if they met the following inclusion criteria: 18-45 years of age; no history of chronic illness; a normal physical examination; human immunodeficiency virus antibody negative, hepatitis B surface antigen negative, and hepatitis C antibody negative; no stool occult blood; and normal values for complete blood cell count (CBC) with differential, hematocrit, platelet count, serum creatinine, serum aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase, bilirubin, prothrombin time (PT), partial thromboplastin time (PTT), and urinalysis. Female volunteers were required to have a negative urine pregnancy test prior to vaccination and on the day of vaccination and to agree to use contraception or abstain from sexual intercourse for the duration of the study. Volunteers also lacked serological evidence of prior flavivirus infection as defined by hemagglutination-inhibition antibody titer < 1:10 to DEN-1, DEN-2, DEN-3, DEN-4, St. Louis encephalitis virus, Japanese encephalitis virus, or yellow fever virus and a plaque-reduction neutralization titer < 1:10 to DEN-4 and yellow fever virus.

[0196] Studies in Humans. Volunteers were immunized in three successive cohorts of four, six, and ten volunteers to assess the safety of the vaccine. In this study, an illness was defined as the following: dengue virus infection associated with a platelet count of < 90,000/mm^3; serum ALT > 4 times normal; oral temperature >38°C for > 2 successive days; or headache and/or myalgia lasting > 2 successive days. Systemic illness was defined as the occurrence of fever >38°C for > 2 consecutive days, or any 2 of the following for at least two consecutive study days: headache, malaise, anorexia, and myalgia/arthralgia. The trials were conducted between October and April, a time of low mosquito prevalence, to reduce the risk of transmission of vaccine virus from the volunteers to the community.

[0197] On the day of vaccination, vaccine candidate 2AΔ30 was diluted to 5.3 $\log_{10}$PFU/ml in sterile saline for injection, USP, and each volunteer was injected subcutaneously with a 0.5 ml containing 5.0 $\log_{10}$PFU of vaccine into the left deltoid region. Volunteers were given a home diary card on which they were to record their temperature twice daily for days 0-5 post-vaccination. The volunteers returned to the clinic each day for examination by a physician and their diary cards were reviewed. The injection site was evaluated for erythema, induration, and tenderness. Clinical signs and symptoms such as headache, rash, petechiae, lymphadenopathy, hepatomegaly, abdominal tenderness, anorexia, nausea, fatigue, myalgia, arthralgia, eye pain, and photophobia were assessed daily. Symptoms were graded as mild (no need for treatment or a change in activity), moderate (treatment needed or change in activity noted, yet still able to continue daily activity) or severe (confined to bed). Blood was drawn for CBC with differential and for virus quantitation on days 0, 2 and 4. Volunteers were admitted to the inpatient unit at the CIR on the sixth day after immunization. The study physician evaluated all volunteers each day by physical examination and interview. The volunteers had their blood pressure, pulse, and temperature recorded four times a day. Blood was drawn each day for CBC with differential and for virus quantitation and every other day for ALT measurement. Volunteers were confined to the inpatient unit until discharge on study day 15. On study days 28 and 42, volunteers returned for physical examination and blood was drawn for virus quantitation (day 28) and for serum antibody measurement (day 28 and 42).

[0198] **Virus quantitation and amplification.** Serum was obtained for detection of viremia and titration of virus in positive specimens. For these purposes 8.5 ml of blood was collected in a serum separator tube and incubated at room temperature for less than 30 min. Serum was decanted into 0.5 ml aliquots, rapidly frozen in a dry ice/ethanol bath and stored at -70°C. Serum aliquots were thawed and serial 10-fold dilutions were inoculated onto Vero cell monolayer cultures in 24-well plates. After one hour incubation at room temperature, the monolayers were overlaid with 0.8% methylcellulose in OptiMEM (Life Technologies) supplemented with 5% fetal bovine serum (FBS). Following incubation at 37°C for four days, virus plaques were visualized by immunoperoxidase staining. Briefly, cell monolayers were fixed in 80% methanol for 30 min and rinsed with antibody buffer (5%

nonfat milk in phosphate buffered saline). Rabbit polyclonal DEN-4 antibodies were diluted 1:1000 in antibody buffer and added to each well followed by a one hr incubation at 37°C. Primary antibody was removed and the cell monolayers were washed twice with antibody buffer. Peroxidase-labelled goat-anti-rabbit IgG (KPL, Gaithersburg, MD) was diluted 1:500 in antibody buffer and added to each well followed by a one hr incubation at 37°C. Secondary antibody was removed and the wells were washed twice with phosphate buffered saline. Peroxidase substrate (4 chloro-1-napthol in H_2O_2) was added to each well and visible plaques were counted.

[0199] For amplification of virus in serum samples, a 0.3 ml aliquot of serum was inoculated directly onto a single well of a 6-well plate of Vero cell monolayers and incubated at 37°C for 7 days. Cell culture fluid was then assayed for virus by plaque assay as described above.

[0200] **Serology.** Hemagglutination-inhibition (HAI) assays were performed as previously described (Clarke, D.H. & Casals, J. 1958 *Am J Trop Med Hyg* 7:561-73). Plaque-reduction neutralization titers (PRNT) were determined by a modification of the technique described by Russell (Russell, P.K. *et al.* 1967 *J Immunol* **99**:285-90). Briefly, test sera were heat inactivated (56°C for 30 min) and serial 2-fold dilutions beginning at 1:10 were made in OptiMEM supplemented with 0.25% human serum albumin. rDEN4Δ30 virus, diluted to a final concentration of 1000 PFU/ml in the same diluent, was added to equal volumes of the diluted serum and mixed well. The virus/serum mixture was incubated at 37°C for 30 min. Cell culture medium was removed from 90% confluent monolayer cultures of Vero cells on 24-well plates and 50 µl of virus/serum mixture was transferred onto duplicate cell monolayers. Cell monolayers were incubated for 60 min at 37°C and overlaid with 0.8% methylcellulose in OptiMEM supplemented with 2% FBS. Samples were incubated at 37°C for 4 days after which plaques were visualized by immunoperoxidase staining as described above, and a 60% plaque-reduction neutralization titer was calculated.

[0201] **Studies in rhesus monkeys.** Evaluation of the replication and immunogenicity of wt virus 814669, and recombinant viruses 2A wt, 2AΔ30 (vaccine lot), rDEN4, and rDEN4Δ30 in juvenile rhesus monkeys was performed as previously described (Men R. *et al.* 1996 *J Virol* **70**:3930-7). Briefly, dengue virus seronegative monkeys were

injected subcutaneously with 5.0 $\log_{10}$ PFU of virus diluted in L-15 medium (Quality Biological, Gaithersburg, MD) containing SPG buffer. A dose of 1 ml was divided between two injections in each side of the upper shoulder area. Monkeys were observed daily and blood was collected on days 0 - 10 and 28, and processed for serum, which was stored frozen at -70°C. Titer of virus in serum samples was determined by plaque assay on Vero cells as described above. Neutralizing antibody titers were determined for the day 28 serum samples as described above. A group of monkeys inoculated with either 2AΔ30 (n = 4) or wt virus 814669 (n = 8) were challenged on day 42 with a single dose of 5.0 $\log_{10}$ PFU/ml wt virus 814669 and blood was collected for 10 days. Husbandry and care of rhesus monkeys was in accordance with the National Institutes of Health guidelines for the humane use of laboratory animals.

[0202] Construction and characterization of DEN-4 wild type and deletion mutant viruses. The nucleotide and deduced amino acid sequences of the previously described wt 814669 virus, the DEN-4 2A wt virus derived from it (designated 2A wt), and the 2AΔ30 vaccine candidate derived from 2A wt virus were first determined. Sequence analysis showed that the wt 814669 virus used in this study had apparently accumulated 2 missense mutations (nucleotides 5826 and 7630) and 3 silent mutations during its passage and amplification since these mutations were not described in previously published reports of the viral sequence (GenBank accession number M14931) and were not present in the 2A cDNA derived from the virus. Sequence comparison between viruses 2A wt and vaccine lot 2AΔ30 revealed that 2AΔ30 accumulated 2 missense mutations (nucleotides 7153 and 8308) and also confirmed the presence of the Δ30 mutation (nucleotides 10478 - 10507) as well as an additional deletion of nucleotide 10475, which occurred during the original construction of the Δ30 mutation (Men, R. *et al.* 1996 *J Virol* **70**:3930-7). This sequence analysis revealed significant sequence divergence between the biologically-derived wt 814669 virus and its recombinant 2A wt derivative and between the 2A wt and 2AΔ30 virus. Since the 2A wt and 2AΔ30 viruses differed at nucleotides other than the deletion mutation, the attenuation phenotype previously reported for 2AΔ30 (Men, R. *et al.* 1996 *J Virol* **70**:3930-7) could not be formally ascribed solely to the Δ30 mutation and may have been specified by the mutations at nucleotides 7153, 8308, 10475, or the Δ30 deletion.

[0203] To determine whether the Δ30 mutation was responsible for the observed attenuation of 2AΔ30, a second pair of viruses, one with and one without the Δ30 mutation, were produced for evaluation in monkeys. A new DEN-4 cDNA vector construct, designated p4, was derived from the 2A-*Xho*I cDNA clone and translationally-silent mutations were introduced to add or ablate several restriction enzyme sites. These sites were added to facilitate the future genetic manipulation of this DEN-4 wt cDNA by the introduction of other attenuating mutations if needed. The sequence of the genomic region of the p4 cDNA plasmid was identical to that of the 2A wt virus except for the engineered restriction site changes and a point mutation at nucleotide 2440 which was introduced during the original mutagenesis of the 2A cDNA plasmid to create the *Xho*I site (Bray, M. & Lai, C.J. 1991 *PNAS USA* **88**:10342-6). The Δ30 mutation and the neighboring deletion at nucleotide 10475 were co-introduced into the p4 plasmid by replacing a short restriction fragment with one derived from the cDNA clone of 2AΔ30. RNA transcripts derived from the p4 cDNA clone and from its Δ30 derivative each yielded virus (designated rDEN4 wt and rDEN4Δ30, respectively) following transfection of Vero cells. Sequence analysis of the rDEN4 virus revealed that during its passage and amplification in Vero cells it accumulated 2 missense mutations (nucleotides 4353 and 6195), a silent mutation (nucleotide 10157), and a point mutation in the 3′ untranslated region (nucleotide 10452). In addition to containing the Δ30 and the accompanying deletion at nucleotide 10475, rDEN4Δ30 had also accumulated a missense mutation (nucleotide 7163) and a silent mutation (nucleotide 7295).

[0204] Parental wt 814669 virus and recombinant viruses 2A wt, 2AΔ30, rDEN4, and rDEN4Δ30 each replicate in Vero cells to a titer exceeding 7.0 $\log_{10}$PFU/ml, and their replication is not temperature sensitive at 39°C.

[0205] **Virus replication, immunogenicity, and efficacy in monkeys.** Groups of rhesus monkeys were inoculated with wt DEN-4 814669, 2A wt, rDEN4, 2AΔ30 and rDEN4Δ30 to assess the level of restriction of replication specified by the Δ30 mutation. Serum samples were collected daily and titer of virus present in the serum was determined by plaque enumeration on Vero cell monolayer cultures. Monkeys inoculated with wt 814669 virus or its recombinant counterparts, 2A wt or rDEN4, were viremic for 3 to 4 days with a mean peak virus titer of nearly 2 $\log_{10}$PFU/ml. Monkeys inoculated with virus 2AΔ30 or

rDEN4Δ30 had a lower frequency of viremia (83% and 50%, respectively), were viremic for only about 1 day, and the mean peak titer was 10-fold lower. Monkeys inoculated with DEN-4 814669, 2A wt, or rDEN4 viruses developed high levels of neutralizing antibody, with mean titers between 442 and 532, consistent with their presumed wild type phenotype. Monkeys inoculated with 2AΔ30 or rDEN4Δ30 developed a lower level of neutralizing antibody, with mean titers of 198 and 223, respectively. The decrease in neutralizing antibody titer in response to 2AΔ30 and rDEN4Δ30 is consistent with the attenuation phenotype of these viruses. Monkeys inoculated with either 2AΔ30 (n = 4) or wt 814669 virus (n = 8) were challenged after 42 days with wt virus 814669. Dengue virus was not detected in any serum sample collected for up to 10 days following virus challenge, indicating that these monkeys were completely protected following immunization with either wt virus or vaccine candidate 2AΔ30.

[0206] Since DEN-4 814669, 2A wt, and rDEN4 each manifest the same level of replication and immunogenicity in rhesus monkeys, it is reasonable to conclude that the identified sequence differences between these presumptive wild type viruses that arose during passage in tissue culture or during plasmid construction do not significantly affect their level of replication in vivo. Similarly, the comparable level of attenuation of 2AΔ30 and rDEN4Δ30 indicates that the mutations shared by these viruses, namely, the Δ30 mutation and its accompanying 10475 deletion mutation, are probably responsible for the attenuation of these viruses rather than their incidental sequence differences.

[0207] **Clinical Response to immunization with 2AΔ30.** The 2AΔ30 vaccine candidate was administered subcutaneously at a dose of 10^5 PFU to 20 seronegative volunteers. Each of the vaccinees was infected and the virus was well tolerated by all vaccinees. Viremia was detected in 70% of the vaccinees, was present only at low titer, and did not extend beyond day 11.

[0208] None of the 20 vaccinees reported soreness or swelling at the injection site. Mild erythema (1-3 mm) around the injection site was noted on examination of 8 volunteers 30 minutes post-vaccination which resolved by the next day in 7 of those volunteers and by the third day in the remaining volunteer. Mild tenderness to pressure at the vaccination site was noted in 2 volunteers and lasted a maximum of 48 hours. During

physical examination, ten volunteers (50%) were noted to a have a very mild dengue-like erythematous macular rash (truncal distribution) which occurred with greatest frequency on day 10. None of the volunteers noted the rash themselves, and it was asymptomatic in each instance. Rash was seen only in vaccinees with detectable viremia. Volunteers did not develop systemic illness. Seven volunteers noted an occasional headache that was described as mild, lasting less than 2 hours, and was not present in any volunteer on two consecutive days. One volunteer reported fever of 38.6°C and 38.2°C without accompanying headache, chills, eye pain, photophobia, anorexia, myalgia, or arthralgia as an outpatient the evening of day 3 and day 5, respectively. However, this volunteer was afebrile when evaluated by the study staff on the morning of days 3, 4, 5 and 6. All other temperature measurements recorded by the volunteer or study staff were normal. Although tourniquet tests were not performed, two volunteers were noted to have petechiae at the site of the blood pressure cuff after a blood pressure measurement was performed (one on day 6, the other on days 7 and 10). Both of these volunteers had normal platelet counts at that time and throughout the study.

[0209] Significant hematological abnormalities were not seen in any vaccinee. Three vaccinees with presumed benign ethnic neutropenia manifested an absolute neutrophil count (ANC) below 1500/mm^3. These three volunteers had baseline ANCs which were significantly lower than the remaining 17 volunteers and which did not decrease disproportionately to the other volunteers. Two of the three volunteers who became neutropenic never had detectable viremia. A mild increase in ALT levels was noted in 4 volunteers, and a more significant increase in ALT level (up to 238 IU/L) was noted in one volunteer. These ALT elevations were transient, were not associated with hepatomegaly, and were completely asymptomatic in each of the 5 volunteers. Elevated ALT values returned to normal by day 26 post-vaccination. The volunteer with the high ALT value was also noted to have an accompanying mild elevation in AST on day 14 (10^4 IU/L) which also returned to baseline by day 26 post-vaccination. This volunteer did not have an associated increase in LDH, bilirubin, or alkaline phosphatase levels.

[0210] **Serologic response of humans to immunization with 2AΔ30.** Each of the twenty vaccinees developed a significant rise in serum neutralizing antibody titer against

DEN-4 by day 28. The level of serum neutralizing antibody was similar in viremic (1:662) and non-viremic vaccinees (1:426). The DEN-4 neutralizing antibody titers of both groups had not changed significantly by day 42.

[0211] Genetic stability of the Δ30 mutation. RT-PCR and sequence analysis of viral RNA isolated from serum samples (n = 6) collected from volunteers 6 to 10 days post-vaccination confirmed the presence of the Δ30 mutation and neighboring deletion at nucleotide 10475.

Example 9

[0212] Pharmaceutical Compositions

[0213] Live attenuated dengue virus vaccines, using replicated virus of the invention, are used for preventing or treating dengue virus infection. Additionally, inactivated dengue virus vaccines are provided by inactivating virus of the invention using known methods, such as, but not limited to, formalin or β-propiolactone treatment. Live attenuated or inactivated viruses containing the mutations described above form the basis of an improved vaccine for the prevention or treatment of dengue infection in humans.

[0214] Pharmaceutical compositions of the present invention comprise live attenuated or inactivated dengue viruses, optionally further comprising sterile aqueous or non-aqueous solutions, suspensions, and emulsions. The composition can further comprise auxiliary agents or excipients, as known in the art. See, e.g., Berkow *et al.* eds. 1987 *The Merck Manual*, 15th edition, Merck and Co., Rahway, N.J.; Goodman *et al.* eds. 1990 Goodman and Gilman's *The Pharmacological Basis of Therapeutics*, 8th edition, Pergamon Press, Inc., Elmsford, N.Y.; *Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics*, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, Md. 1987; Osol, A. ed. 1980 *Remington's Pharmaceutical Sciences* Mack Publishing Co, Easton, Pa. pp. 1324-1341; Katzung, ed. 1992 *Basic and Clinical Pharmacology* Fifth Edition, Appleton and Lange, Norwalk, Conn.

[0215] A virus vaccine composition of the present invention can comprise from about 10^2 –10^9 plaque forming units (PFU)/ml, or any range or value therein, where the virus is attenuated. A vaccine composition comprising an inactivated virus can comprise an

amount of virus corresponding to about 0.1 to 50 μg of E protein/ml, or any range or value therein.

[0216] The agents may be administered using techniques well known to those in the art. Preferably, agents are formulated and administered systemically. Suitable routes may include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intradermal, intranasal, or intraocular injections, just to name a few. For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as saline, phosphate buffered saline, Tris buffered saline, Hank's buffered saline, growth media such as Eagle's Minimum Essential Medium (MEM), and the like.

[0217] When a vaccine composition of the present invention is used for administration to an individual, it can further comprise salts, buffers, adjuvants, or other substances which are desirable for improving the efficacy of the composition. Adjuvants useful with the invention include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides or bacterial cell wall components), such as for example (a) MF59 (International Publication No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE, although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (3) saponin adjuvants, such as Stimulon™ (Cambridge Bioscience, Worcester, MA) may be used or particle generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freunds Adjuvant (CFA) and Incomplete

Freunds Adjuvant (IFA); (5) cytokines, such as interleukins (IL-1, IL-2, etc.), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc.; (6) mucosal adjuvants such as those derived from cholera toxin (CT), pertussis toxin (PT), *E. coli* heat labile toxin (LT), and mutants thereof (see, e.g., International Publication Nos. WO 95/17211, WO 93/13202, and WO 97/02348); and (7) other substances that act as immunostimulating agents to enhance the effectiveness of the composition.

[0218] The pharmacologically active compounds of this invention can be processed in accordance with conventional methods of galenic pharmacy to produce medicinal agents for administration to patients, e.g., mammals including humans.

[0219] The compounds of this invention can be employed in admixture with conventional excipients, i.e., pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, enteral (e.g., oral) or topical application, which do not deleteriously react with the active compounds. Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions, alcohols, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, pentaerythritol fatty acid esters, hydroxy methylcellulose, polyvinyl pyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic substances and the like which do not deleteriously react with the active compounds. They can also be combined where desired with other active agents, e.g., vitamins.

[0220] For parenteral application, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants, including suppositories. Ampoules are convenient unit dosages.

[0221] For enteral application, particularly suitable are tablets, dragees, liquids, drops, suppositories, or capsules. A syrup, elixir, or the like can be used wherein a sweetened vehicle is employed.

[0222] For topical application, there are employed as non-sprayable forms, viscous to semi-solid or solid forms comprising a carrier compatible with topical application

and having a dynamic viscosity preferably greater than water. Suitable formulations include but are not limited to solutions, suspensions, emulsions, creams, ointments, powders, liniments, salves, aerosols, etc., which are, if desired, sterilized or mixed with auxiliary agents, e.g., preservatives, stabilizers, wetting agents, buffers or salts for influencing osmotic pressure, etc. For topical application, also suitable are sprayable aerosol preparations wherein the active ingredient, preferably in combination with a solid or liquid inert carrier material, is packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant, e.g., a freon.

[0223] The vaccine can also contain variable but small quantities of endotoxin, free formaldehyde, and preservative, which have been found safe and not contributing to the reactogenicity of the vaccines for humans.

Example 10

Pharmaceutical Purposes

[0224] The administration of the vaccine composition may be for either a "prophylactic" or "therapeutic" purpose. When provided prophylactically, the compositions are provided before any symptom of dengue viral infection becomes manifest. The prophylactic administration of the composition serves to prevent or attenuate any subsequent infection. When provided therapeutically, the live attenuated or inactivated viral vaccine is provided upon the detection of a symptom of actual infection. The therapeutic administration of the compound(s) serves to attenuate any actual infection. See, e.g., Berkow *et al.* eds. 1987 *The Merck Manual*, 15th edition, Merck and Co., Rahway, N.J.; Goodman *et al.* eds. 1990 *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 8th edition, Pergamon Press, Inc., Elmsford, N.Y.; *Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics*, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, Md. 1987; Katzung, ed. 1992 *Basic and Clinical Pharmacology*, Fifth Edition, Appleton and Lange, Norwalk, Conn.

[0225] A live attenuated or inactivated vaccine composition of the present invention may thus be provided either before the onset of infection (so as to prevent or attenuate an anticipated infection) or after the initiation of an actual infection.

[0226] The vaccines of the invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby live attenuated or inactivated viruses are combined in a mixture with a pharmaceutically acceptable vehicle. A composition is said to be a "pharmacologically acceptable vehicle" if its administration can be tolerated by a recipient patient. Suitable vehicles are well known to those in the art, e.g., in Osol, A. ed. 1980 *Remington's Pharmaceutical Sciences* Mack Publishing Co, Easton, Pa. pp. 1324-1341.

[0227] For purposes of administration, a vaccine composition of the present invention is administered to a human recipient in a therapeutically effective amount. Such an agent is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. A vaccine composition of the present invention is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient that generates a host immune response against at least one dengue serotype, stimulates the production of neutralizing antibodies, or leads to protection against challenge.

[0228] The "protection" provided need not be absolute, i.e., the dengue infection need not be totally prevented or eradicated, if there is a statistically significant improvement compared with a control population or set of patients. Protection may be limited to mitigating the severity or rapidity of onset of symptoms of the dengue virus infection.

Example 11

[0229] Pharmaceutical Administration

[0230] A vaccine of the present invention may confer resistance to one or more dengue serotypes by immunization. In immunization, an live attenuated or inactivated vaccine composition is administered prophylactically, according to a method of the present invention. In another embodiment a live attenuated or inactivated vaccine composition is administered therapeutically, according to a different method of the present invention.

[0231] The present invention thus includes methods for preventing or attenuating infection by at least one dengue serotype. As used herein, a vaccine is said to prevent or attenuate a disease if its administration results either in the total or partial attenuation (i.e.,

suppression) of a symptom or condition of the disease, or in the total or partial immunity of the individual to the disease.

[0232] At least one live attenuated or inactivated dengue virus, or composition thereof, of the present invention may be administered by any means that achieve the intended purpose, using a pharmaceutical composition as previously described.

[0233] For example, administration of such a composition may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, oral or transdermal routes. Parenteral administration can be by bolus injection or by gradual perfusion over time. A preferred mode of using a pharmaceutical composition of the present invention is by intramuscular, intradermal or subcutaneous application. See, e.g., Berkow *et al.* eds. 1987 *The Merck Manual* 15th edition, Merck and Co., Rahway, N.J.; Goodman *et al.* eds. 1990 *Goodman and Gilman's The Pharmacological Basis of Therapeutics,* 8th edition, Pergamon Press, Inc., Elmsford, N.Y.; *Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics*, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, Md. 1987; Osol, A. ed. 1980 *Remington's Pharmaceutical Sciences*, Mack Publishing Co, Easton, Pa. pp. 1324-1341; Katzung, ed. 192 *Basic and Clinical Pharmacology*, Fifth Edition, Appleton and Lange, Norwalk, Conn.

[0234] A typical regimen for preventing, suppressing, or treating a dengue virus related pathology, comprises administration of an effective amount of a vaccine composition as described herein, administered as a single treatment, or repeated as enhancing or booster dosages, over a period up to and including between one week and about 24 months, or any range or value therein.

[0235] It will be appreciated that the actual preferred amounts of active compound in a specific case will vary according to the specific compound being utilized, the compositions formulated, the mode of application, and the particular situs and organism being treated. Dosages for a given host can be determined using conventional considerations, e.g., by customary comparison of the differential activities of the subject compounds and of a known agent, e.g., by means of an appropriate, conventional pharmacological protocol.

[0236] The dosage of a live attenuated virus vaccine for a mammalian (e.g., human) subject can be from about 10^3 -10^7 plaque forming units (PFU)/kg, or any range or value therein. The dose of inactivated vaccine can range from about 0.1 to 50 μg of E protein. However, the dosage should be a safe and effective amount as determined by conventional methods, using existing vaccines as a starting point.

[0237] The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

Table 1. Susceptibility of mice to intracerebral DEN4 infection is age-dependent[a]

Virus	Mean virus titer (log_{10}PFU/g brain) ± SE following inoculation at indicated age (days)		
	7	14	21
2A-13	>6.0	4.0 ± 0.2	3.1 ± 0.2
rDEN4	>6.0	3.3 ± 0.4	3.3 ± 0.2
rDEN4Δ30	>6.0	3.6 ± 0.2	2.8 ± 0.3

[a] Groups of 4 or 5 Swiss Webster mice were inoculated intracerebrally with 10^5 PFU virus in a 30 µl inoculum. After 5 days, brains were removed, homogenized and titered in Vero cells. SE = Standard error.

TABLE 2 Temperature-sensitive (*ts*) and mouse brain attenuation (*att*) phenotypes of 5-FU mutant DEN4 viruses.

Phenotype	Virus	Mean virus titer (log_{10}PFU/ml) at indicated temp. (°C)										Virus replication in suckling mice[b]		
		Vero cells					HuH-7 cells					n	Mean titer ± SE (log_{10}PFU/g brain)	Mean log_{10} reduction from wt[d]
		35	37	38	39	Δ[a]	35	37	38	39	Δ			
wt (not *ts*)	2A-13	7.8	7.7	7.6	7.3	0.5	7.8	7.7	7.4	6.4	1.4	66	6.6 ± 0.1[c]	-
	rDEN4	6.5	6.4	6.4	6.0	0.5	7.1	6.7	6.0	5.5	1.6	66	6.1 ± 0.1[c]	-
	rDEN4Δ30	6.3	6.1	6.1	5.7	0.6	6.9	6.3	5.9	4.7	2.2	64	5.6 ± 0.1[c]	0.5
ts in Vero and HuH-7 cells	695	6.2	6.0	5.2	2.6[e]	3.6	6.5	5.5	3.8	≤1.6	>4.9	6	3.0 ± 0.2	**3.2**
	816	6.8	6.4	5.8	3.9	2.9	7.5	6.2	5.5	3.1	4.4	6	3.3 ± 0.4	**2.9**
	773	7.4	6.6	6.0	3.1	4.3	7.7	6.1	5.2	3.1	4.6	12	3.7 ± 0.1	**2.6**
	489	7.3	6.6	6.1	3.3	4.0	7.3	6.7	5.4	3.0	4.3	6	4.5 ± 0.5	**2.3**
	173	7.0	6.1	3.2	2.9	4.1	7.0	3.2	3.0	2.1	4.9	18	4.7 ± 0.4	**2.2**
	509	6.2	5.8	5.5	3.4	2.8	6.5	6.1	4.5	≤1.6	>4.9	6	4.9 ± 0.3	**1.9**
	938	7.1	6.5	5.6	3.1	4.0	7.2	6.4	5.6	3.1	4.1	6	5.1 ± 0.2	**1.7**
	1033	6.7	6.0	5.9	4.1	2.6	6.9	5.6	4.7	≤1.6	>5.3	12	4.7 ± 0.2	**1.7**
	239	7.6	6.8	5.6	3.3	4.3	7.6	6.7	4.7	2.5	5.1	12	4.7 ± 0.3	**1.5**
	793	6.5	5.8	5.3	4.0	2.5	7.2	6.8	5.6	≤1.6	>5.6	6	5.4 ± 0.3	1.4
	759	7.2	6.9	6.4	4.7	2.5	7.5	6.8	6.3	3.1	4.4	12	5.1 ± 0.1	1.4
	718	6.1	5.9	5.3	3.5	2.6	7.0	6.5	5.7	1.7	5.3	12	5.0 ± 0.3	1.4
	473	6.7	6.3	5.4	2.0	4.7	7.2	6.7	3.7	1.9	5.3	12	5.1 ± 0.3	1.2
ts in only HuH-7 cells	686	7.0	6.7	6.7	6.4	0.6	7.3	6.8	6.4	2.2	5.1	12	2.7 ± 0.2	**3.8**
	967	6.8	6.4	6.4	5.1	1.7	6.8	6.4	5.4	≤1.6	>5.2	6	3.6 ± 0.2	**2.9**
	992	7.3	7.1	6.8	5.9	1.4	7.4	6.9	5.0	≤1.6	>5.8	6	3.8 ± 0.1	**2.7**
	571	6.9	7.0	6.4	4.6	2.3	7.0	6.3	5.2	≤1.6	>5.4	6	4.4 ± 0.4	**2.4**
	605	7.6	7.5	7.1	6.9	0.7	7.8	7.2	6.8	≤1.6	>6.2	12	4.5 ± 0.4	**2.1**
	631	7.1	6.9	6.8	5.0	2.1	7.3	7.1	6.5	≤1.6	>5.7	12	4.8 ± 0.3	**1.9**
	1175	7.4	7.1	6.9	5.3	2.1	7.6	6.5	4.7	3.3	4.3	12	4.7 ± 0.2	**1.7**

[a] Reduction in titer (log_{10}PFU/ml) at 39°C compared to titer at permissive temperature (35°C).

[b] Groups of 6 suckling mice were inoculated i.c. with 10^4 PFU virus in a 30 µl inoculum. Brains were removed 5 days later, homogenized, and titered in Vero cells.

[c] Average of 11 experiments with a total of 64 to 66 mice per group.

[d] Determined by comparing mean viral titers of mice inoculated with mutant virus and the 2A-13 wt control in the same experiment (n= 6 or 12).

[e] Underlined values indicate a 2.5 or 3.5 log_{10}PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temp when compared to titer at permissive temp (35°C).

Table 3. Nucleotide and amino acid differences of the 5-FU mutant viruses which are *ts* in both Vero and HuH-7 cells.

Virus	Mutations in UTR or coding region that result in an amino acid substitution				Mutations in coding region that do not result in an amino acid substitution		
	Nucleotide position	Gene/ region	Nucleotide change	Amino Acid change[b]	Nucleotide position	Gene	Nucleotide change
173[a]	7163	NS4B	A > C	L2354F	10217	NS5	A > U
	7849	NS5	A > U	N2583I			
	8872	NS5	A > G	K2924R			
239[a]	4995	NS3	U > C	S1632P	7511	NS4B	G > A
					10070	NS5	U > C
473[a]	4480	NS2B	U > C	V1460A	7589	NS5	G > A
	4995	NS3	U > C	S1632P	10070	NS5	U > C
489[a]	4995	NS3	U > C	S1632P	2232	E	U > C
					3737	NS2A	C > U
509[a]	4266	NS2B	A > G	S1389G	none		
	8092	NS5	A > G	E2664G			
695	40	5' UTR	U > C	n/a	1391	E	A > G
	1455	E	G > U	V452F			
	6106	NS3	A > G	E2002G			
	7546	NS4B	C > U	A2482V			
718	2280	E	U > C	F727L	none		
	4059	NS2A	A > G	I1320V			
	4995	NS3	U > C	S1632P			
	7630	NS5	A > G	K2510R			
	8281	NS5	U > C	L2727S			
759[a]	4995	NS3	U > C	S1632P	none		
	8020	NS5	A >U	N2640I			
773[a]	4995	NS3	U > C	S1632P	none		
793	1776	E	G > A	A559T	5771	NS3	U > C
	2596	NS1	G > A	R832K	7793	NS5	U > A
	2677	NS1	A > G	D859G			
	4387	NS2B	C > U	S1429F			
816[a]	4995	NS3	U > C	S1632P	6632	NS4A	G > A
	7174	NS4B	C > U	A2358V	6695	NS4A	G > A

Virus	Mutations in UTR or coding region that result in an amino acid substitution				Mutations in coding region that do not result in an amino acid substitution		
	Nucleotide position	Gene/ region	Nucleotide change	Amino Acid change[b]	Nucleotide position	Gene	Nucleotide change
938[a]	3442	NS1	A > G	E1114G	747	prM	U > C
	4995	NS3	U > C	S1632P	4196	NS2b	U > C
	10275	3' UTR	A > U	n/a	6155	NS3	G > A
1033[a]	4907	NS3	A > U	L1602F	548	prM	C > U
	8730	NS5	A > C	N2877H			
	9977	NS5	G > A	M3292I			

[a] Viruses that contain mutation(s) resulting in an a.a. substitution in only a NS gene(s) and/or nucleotide substitutions in the UTRs are indicated; i.e. no a.a. substitutions are present in the structural proteins (C-prM-E).

[b] Amino acid position in DEN4 polyprotein beginning with the methionine residue of the C protein (nt 102-104) as residue #1. Wild-type amino acid on left of amino acid position; mutant amino acid on right.

Table 4. Nucleotide and amino acid differences of the 5-FU mutant viruses which are *ts* in only HuH-7 cells.

Virus	Mutations in UTR or coding region that result in an amino acid substitution				Mutations in coding region that do not result in an amino acid substitution		
	Nucleotide position	Gene/ region	Nucleotide change	Amino acid change[b]	Nucleotide position	Gene	Nucleotide change
571	586	prM	U > C	V162A	6413	NS4A	U > C
	7163	NS4B	A > U	L2354F			
	7947	NS5	G > A	G2616R			
605	1455	E	G > U	V452F	none		
	7546	NS4B	C > U	A2482V			
631	595	prM	A > G	K165R	1175	E	G > A
	6259	NS3	U > C	V2053A	5174	NS3	A > G
	7546	NS4B	C > U	A2482V			
686[a]	3575	NS2A	G > A	M1158I	4604	NS3	A > G
	4062	NS2A	A > G	T1321A	7937	NS5	A > G
	7163	NS4B	A > U	L2354F			
967	2094	E	G > C	A665P	4616	NS3	C > U
	2416	E	U > C	V772A			
	7162	NS4B	U > C	L2354S			
	7881	NS5	G > A	G2594S			
992[a]	5695	NS3	A > G	D1865G	3542	NS2A	A > G
	7162	NS4B	U > C	L2354S			
1175[a]	7153	NS4B	U > C	V2351A	6167	NS3	U > C
	10186	NS5	U > C	I3362T	10184	NS5	G > A
	10275	3' UTR	A > U	n/a			

[a] Viruses that contain mutation(s) resulting in an a.a. substitution in only a NS gene(s) and/or nucleotide substitutions in the UTRs are indicated; i.e. no a.a. substitutions are present in the structural proteins.

[b] Amino acid position in DEN4 polyprotein beginning with the methionine residue of the C protein (nt 102-104) as residue #1. Wild-type amino acid on left of amino acid position; mutant amino acid on right.

TABLE 5. Mutations which are represented in multiple 5-FU mutant DEN4 viruses.

Nucleotide position	Gene/ region	Nucleotide change	Amino acid change	Number of viruses with "sister" mutations
1455	E	G > U	val > phe	2
4995	NS3	U > C	ser > pro	8
7162	NS4B	U > C	leu > ser	2
7163	NS4B	A > U or C	leu > phe	3
7546	NS4B	C > U	ala > val	3
10275	3' UTR	A > U	n/a[a]	2

[a] not applicable

Table 6. Addition of *ts* mutation 4995 to rDEN4Δ30 confers a *ts* phenotype and further attenuates its replication in suckling mouse brain.

Virus	Mean virus titer (log_{10}PFU/ml) at indicated temp (°C)										Replication in suckling mice[b]	
	Vero cells					HuH-7 cells					Mean virus titer ± SE (log_{10}PFU/g brain)	Mean log_{10} reduction from *wt*[c]
	35	37	38	39	Δ[a]	35	37	38	39	Δ		
2A-13	7.1	7.1	6.9	6.8	0.3	7.4	7.3	6.7	6.4	1.0	6.5 ± 0.1	-
rDEN4	7.0	6.8	6.6	6.4	0.6	7.5	7.3	6.7	6.4	1.1	6.1 ± 0.2	-
rDEN4Δ30	7.0	6.7	6.2	6.2	0.8	7.5	7.0	6.5	5.1	2.4	5.9 ± 0.1	0.2
rDEN4-4995	5.7	4.9	3.6	≤1.6	>4.1	6.4	5.7	4.0	≤1.6	>4.8	3.2 ± 0.2	**2.9**
rDEN4Δ30-4995	5.9	4.9	3.9	≤1.6[d]	>4.3	6.4	5.6	4.4	≤1.6	>4.8	3.0 ± 0.3	**3.1**

[a] Reduction in titer (log_{10}PFU/ml) at 39°C compared to titer at permissive temperature (35°C).

[b] Groups of 6 suckling mice were inoculated i.c. with 10^4 PFU virus in a 30 µl inoculum. Brains were removed 5 days later, homogenized, and titered in Vero cells. The limit of detection is 2.0 log_{10}PFU/g brain.

[c] Determined by comparing mean viral titers of mice inoculated with sample virus and rDEN4 control.

[d] Underlined values indicate a 2.5 or 3.5 log_{10}PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temperature when compared to permissive temperature.

Table 7. Temperature-sensitive (*ts*) and mouse brain attenuation (*att*) phenotypes of 5-FU DEN4 mutant viruses which exhibit a small plaque (*sp*) phenotype.

Phenotype				Virus	Mean virus titer (log_{10}PFU/ml) at indicated temp (°C)										Replication in suckling mice[b]		
sp		*ts*			Vero cells					HuH-7 cells						Mean virus titer ± SE (log_{10}PFU/g brain)	Mean log_{10} reduction from *wt*[d]
Vero	HuH-7	Vero	HuH-7		35	37	38	39	Δ[a]	35	37	38	39	Δ	n		
—	—	—	—	2A-13	7.9	7.5	7.7	7.2	0.7	7.9	7.7	7.3	6.9	1.0	66	6.6 ± 0.1[c]	—
—	—	—	—	rDEN4	7.9	7.6	7.7	7.3	0.6	8.1	7.6	7.5	6.7	1.4	66	6.1 ± 0.1[c]	—
—	—	—	—	rDEN4Δ30	7.3	6.6	6.6	6.1	1.2	7.3	7.2	6.9	5.9	1.4	64	5.6 ± 0.1[c]	0.5
+	+	+	+	574	6.6[x]	5.5	3.8	≤1.6[e]	≥5.0	6.6[x]	4.9	5.0	≤1.6	≥5.0	6	2.1 ± 0.1	**5.1**
+	+	+	+	1,269	5.3[x]	4.8	3.9	≤1.6	≥3.7	4.0[x]	2.4	2.0	<1.6	≥2.4	6	2.7 ± 0.2	**4.1**
+	+	+	+	1,189	6.3[x]	5.2	4.5	3.8	2.5	5.5[x]	3.7	2.3	≤1.6	≥3.9	12	3.2 ± 0.4	**3.7**
+	+	—	—	569	5.8[x]	5.6	5.6	3.7	2.1	6.2[x]	6.0	5.7	5.0	1.2	12	1.9 ± 0.1	**4.6**
+	+	—	—	761	5.0[x]	4.7	4.2	2.7	2.3	5.6[x]	5.3	4.5	2.6	3.0	12	2.0 ± 0.1	**4.2**
—	+	+	+	506	7.0	6.8	5.6	2.6	4.4	6.7[x]	4.3	≤1.6	2.0	4.7	6	2.2 ± 0.1	**4.7**
—	+	+	+	1,136	5.1	4.2	2.6	≤1.6	≥3.5	5.7[x]	3.0	3.0	≤1.6	≥4.1	6	2.9 ± 0.3	**4.5**
—	+	+	+	1,029	6.9	5.8	5.8	2.9	4.0	7.0[x]	5.8	5.2	2.5	4.5	6	2.2 ± 0.1	**4.2**
—	+	+	+	1,081	6.9	5.8	4.7	3.9	3.0	5.8[x]	4.1	3.3	1.9	3.9	12	2.6 ± 0.2	**3.9**
—	+	+	+	529	6.9	6.5	5.9	4.0	2.9	7.1[x]	5.3	4.4	≤1.6	≥5.5	6	3.1 ± 0.7	**3.8**
—	+	+	+	1,114	6.7	6.4	6.2	2.5	4.2	5.7[x]	3.0	2.9	1.9	3.8	6	2.7 ± 0.3	**3.7**
—	+	+	+	922	7.3	7.2	6.8	3.8	3.5	7.4[x]	5.3	4.1	3.0	4.4	12	3.5 ± 0.1	**2.9**
—	+	+	+	311	6.9	5.9	4.3	1.5	5.4	7.1[x]	5.4	3.6	≤1.6	≥5.5	12	6.1 ± 0.3	0.9
—	+	+	+	326	6.6	5.7	4.5	3.1	3.5	7.0[x]	5.5	4.1	2.0	5.0	6	6.0 ± 0.1	0.9
—	+	—	+	1,104	7.1	6.8	6.8	6.1	1.0	7.2[x]	6.4	5.8	2.8	4.4	6	2.2 ± 0.1	**4.7**
—	+	—	+	952	7.1	7.0	6.7	5.6	1.5	7.3[x]	6.3	5.6	3.0	4.3	6	2.4 ± 0.3	**4.5**
—	+	—	+	738	6.5	6.0	5.9	5.7	0.8	6.9[x]	6.1	5.0	3.1	3.8	12	4.4 ± 0.4	**2.3**
—	+	—	+	1,083	7.4	7.3	7.4	5.8	1.6	7.4[x]	6.6	4.5	≤1.6	≥5.8	12	4.5 ± 0.4	**2.0**
—	+	—	—	1,096	7.5	7.1	6.9	5.5	2.0	7.5[x]	6.6	5.6	4.8	2.7	6	2.9 ± 0.2	**3.5**
—	+	—	—	1,021	7.0	6.9	6.6	6.3	0.7	6.9[x]	5.7	4.4	4.0	2.9	6	3.9 ± 0.6	**2.6**

—	+	—	—	1,023	6.6	6.4	6.0	5.8	0.8	6.1[x]	5.6	4.7	3.3	2.8	12	4.2 ± 0.3	**2.3**
—	+	—	—	1,012	7.5	7.1	7.0	5.7	1.8	7.4[x]	6.8	6.8	5.6	1.8	6	6.1 ± 0.1	0.8

[a] Reduction in mean virus titer (log_{10}PFU/ml) at 39°C compared to permissive temperature (35°C).

[b] Groups of 6 suckling mice were inoculated i.c. with 10^4 PFU virus. Brains were removed 5 days later, homogenized, and titered in Vero cells.

[c] Average of 11 experiments with a total of 64 to 66 mice per group.

[d] Determined by comparing mean viral titers of mice inoculated with mutant virus and concurrent 2A-13 wild type (*wt*) virus control (n = 6 or 12).

[e] Underlined values indicate a 2.5 or 3.5 log_{10}PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temperature when compared to permissive temperature (35°C).

[x] Small plaque size at 35°C; small plaques have a diameter of <1.0 mm compared to wild type plaque diameter of 1.5 - 2.0 mm in Vero cells, or a diameter of <0.4 mm compared to wild type plaque diameter of 0.75 to 1.0 mm in HuH-7 cells.

Table 8. Viruses with both *ts* and *sp* phenotypes are more restricted in replication in mouse brain than those with only a *ts* phenotype.

Cell culture phenotype	Number of viruses	Mean $\log_{10}$ reduction in virus titer from control[b, c]
ts[a]	20	2.1 ± 0.2
sp	6	3.0 ± 0.6
ts / sp	16	3.5 ± 0.3

[a] 20 *ts* mutant viruses without an *sp* phenotype were previously described (Example 1).

[b] Determined by comparing mean viral titers of groups of mice inoculated with mutant virus and concurrent 2A-13 parallel-passaged control virus.

[c] Significant difference between *ts* group and *ts / sp* group, Tukey-Kramer test ($P < 0.05$)

Table 9. Nucleotide and amino acid differences of the 5-FU mutant DEN4 viruses which produce small plaques in both Vero and HuH-7 cells.

Virus	Mutations in UTR or in coding regions that result in an amino acid substitution				Mutations in coding regions that do not result in an amino acid substitution		
	Nucleotide position	Gene/ region	Nucleotide change	Amino acid change[b]	Nucleotide position	Gene	Nucleotide change
569	826	prM	G > A	R242K	1946	E	C > U
	832	prM	C > U	P244L			
	7546	NS4B	C > U	A2482V			
	10275	3' UTR	A > U	n/a			
	10279	3' UTR	A > U	n/a			
574	1455	E	G > U	V452F	1349	E	C > U
	1963	E	U > C	V621A			
	3880	NS2A	A > G	K1260R			
	7546	NS4B	C > U	A2482V			
	7615	NS5	A > G	N2505S			
	10413	3' UTR	A > G	n/a			
761	424	C	U > C	I108T	none		
	2280	E	U > C	F727L			
	7131	NS4B	A > G	T2344A			
	7486	NS4B	A > G	N2462S			
1189a	3303	NS1	A > G	R1068G	6719	NS4A	U > C
	4812	NS3	G > A	V1571I			
	5097	NS3	G > A	D1666N			
	7182	NS4B	G > A	G2361S			
1269	2112	E	U > C	F671L	542	prM	C > U
	3256	NS1	G > A	G1052E			
	3993	NS2A	U > C	F1298L			
	7183	NS4B	G > U	G2361V			

[a] Virus contains missense mutations in only the non-structural genes.

[b] Amino acid position in DEN4 polyprotein beginning with the methionine residue of the C protein (nt 102-104).
Wild type amino acid on left of amino acid position; mutant amino acid on right.

Table 10. Nucleotide and amino acid differences of the 5-FU mutant DEN4 viruses which produce small plaques in only HuH-7 cells.

Virus	Mutations in UTR or in coding regions that result in an amino acid substitution				Mutations in coding regions that do not result in an amino acid substitution		
	Nucleotide position	Gene/ region	Nucleotide change	Amino acid change[b]	Nucleotide position	Gene	Nucleotide change
311	1519	E	A > G	N473S	6761	NS4A	C > U
	2305	E	G > A	R735K	10070	NS5	U > C
	4896	NS3	G > U	A1599S			
326	1587	E	C > U	P496S	1523	E	G > A
	7546	NS4B	C > U	A2482V	6080	NS3	U > C
					10070	NS5	U > C
506	1455	E	G > U	V452F	3887	NS2A	A > G
	1902	E	G > A	V601M	5789	NS3	G > C
	7546	NS4B	C > U	A2482V			
	10275	3' UTR	A > U	n/a			
529	777	prM	U > C	S226P	none		
	4641	NS3	A > G	I1514V			
	7153	NS4B	U > C	V2351A			
	8245	NS5	U > C	I2715T			
	10279	3' UTR	A > C	n/a			
738[a]	3540	NS2A	G > A	E1147K	none		
	7162	NS4B	U > C	L2354S			
922[a]	4306	NS2B	A > G	N1402S	7736	NS5	G > A
	5872	NS3	C > U	T1924I			
	7163	NS4B	A > U	L2354F			
	10279	3' UTR	A > C	n/a			
952	1449	E	G > U	V450L	none		
	1455	E	G > U	V452F			
	7546	NS4B	C > U	A2482V			
	7957	NS5	U > C	V2619A			
	9543	NS5	A > G	I3148V			
1012	1542	E	A > G	K481E	953	E	A > G
	7162	NS4B	U > C	L2354S	1205	E	G > A
	10542	3' UTR	A > G	n/a	4425	NS2B	U > C
1021	2314	E	U > C	I738T	665	prM	C > A
	3205	NS1	C > U	A1035V	5750	NS3	C > U
	4029	NS2A	U > C	C1310R	9959	NS5	C > U
	7163	NS4B	A > C	L2354F			

Virus	Mutations in UTR or in coding regions that result in an amino acid substitution				Mutations in coding regions that do not result in an amino acid substitution		
	Nucleotide position	Gene/ region	Nucleotide change	Amino acid change[b]	Nucleotide position	Gene	Nucleotide change
	10275	3' UTR	A > U	n/a			
	10279	3' UTR	A > U	n/a			
1023	2283	E	G > A	G728R	1001	E	C > U
	7182	NS4B	G > A	G2361S	1958	E	A > G
					3873	NS2a	U > C
					8486	NS5	C > U
1029	850	prM	C > U	A250V	3867	NS2a	C > U
	3087	NS1	A > G	T996A			
	4891	NS3	U > C	I1597T			
1081[a]	2650	NS1	A > G	N850S	6326	NS3	C > U
	7163	NS4B	A > U	L2354F	9146	NS5	C > U
1083[a]	3702	NS2A	G > A	A1201T	3353	NS1	A > G
	7153	NS4B	U > C	V2351A	6155	NS3	G > A
	10634	3' UTR	U > C	n/a			
1096	892	prM	G > A	R264Q	665	prM	C > A
	7163	NS4B	A > C	L2354F	4427	NS2b	G > A
	8659	NS5	C > U	P2853L			
1104	1692	E	G > A	V531M	none		
	5779	NS3	C > U	A1893V			
	7546	NS4B	C > U	A2482V			
1114	709	prM	A > G	K203R	1076	E	U > C
	3693	NS2A	A > G	I1198V	1182	E	C > U
	4614	NS3	U > C	F1505L	5690	NS3	C > U
	7546	NS4B	C > U	A2482V			
	9942	NS5	A > G	T3281A			
1136[a]	3771	NS2A	A > G	R1224G	5621	NS3	A > G
	4891	NS3	U > C	I1597T			
	10275	3' UTR	A > U	n/a			

[a] Viruses that contain missense mutations in only the non-structural genes and/or mutations in the UTRs.

[b] Amino acid position in DEN4 polyprotein beginning with the methionine residue of the C protein (nt 102-104).
Wild type amino acid on left of amino acid position; mutant amino acid on right.

Table 11. Putative Vero cell adaptation mutations derived from the full set of 5-FU mutant viruses.

Nucleotide position	Gene / region (a.a. #)[b]	5-FU mutant viruses		
		Nucleotide change	Amino acid change	No. of viruses with the mutation
1455	E (452)	G > U	Val > Phe	5
2280	E (727)	U > C	Phe > Leu	2
4891	NS3 (1597)	U > C	Ile > Thr	2
4995	NS3 (1599)	U > C	Ser > Pro	8
7153	NS4B (2351)	U > C	Val > Ala	3
7162	NS4B (2354)	U > C	Leu > Ser	4
7163	NS4B (2354)	A > U or C	Leu > Phe	7
7182	NS4B (2361)	G > A	Gly > Ser	2
7546	NS4B (2482)	C > U	Ala > Val	10
7630	NS5 (2510)	A > G	Lys > Arg	1
10275	3' UTR	A > U	n/a[a]	6
10279	3' UTR	A > C	n/a	4

[a] not applicable

[b] Amino acid position in DEN4 polyprotein beginning with the methionine residue of the C protein (nt 102-104) as residue #1.

Table 12. Mutagenic oligonucleotides used to generate recombinant DEN4 viruses containing single 5-FU mutations.

SEQ ID NO.	Recombinant virus (rDEN4-)	Nucleotide change	Amino acid change	Gene	pUC clone	RE site[a]	Oligonucleotide[b]
23	40	U > C	n/a	5' UTR	pUC-*Nhe*I	*Bsa*WI	CAGTTCCAAAcCGGAAGCTTG
24	2650	A > G	Asn > Ser	NS1	pUC-NS1	*Bsi*WI	CCAACGAGCTAtcgTAcGTTCTCTGGG
25	3303	A > G	Arg > Gly	NS1	pUC-NS1	*Sty*I	GATTGTGACCATgGcGGCCCATCTTTG
26	3442	A > G	Glu > Gly	NS1	pUC-NS1	*Blp*I	GGAGATTAGGCCgcTGAGcGgtAAAGAAGAG
27	3540	G > A	Glu > Lys	NS2A	pUC-NS1	*Bsm*I	GTTTGTGGAAaAATGtcTGAGGAGAA
28	3575	G > A	Met > Ile	NS2A	pUC-NS1	*Ssp*I	CTAGGAAACACATaATATTAGTTGTGG
29	3702	G > A	Ala > Thr	NS2A	pUC-NS2A	*Bgl*I	CAGATCCACCTAaCCATaATGGCAGTG
30	3771	A > G	Arg > Gly	NS2A	pUC-NS2A	*Ava*I	GGAAACTCACcTCggGAGAGACAGC
31	4059	A > G	Ile > Val	NS2A	pUC-NS2A	*Bst*EII	TTGGGTAGAggTcACcGCACTCATCC
32	4062	A > G	Thr > Ala	NS2A	pUC-NS2A	*Bsr*BI	GTAGAAATAgCcGCtCTCATCCTAG
33	4266	A > G	Ser > Gly	NS2B	pUC-NS2A	*Sna*BI	GGCGGCTTACGTaATGgGaGGTAGCTCAGC
34	4306	A > G	Asn > Ser	NS2B	pUC-NS2A	*Alw*NI	CTAGAGAAGGCaGCttctGTGCAGTGG
35	4480	U > C	Val > Ala	NS2B	pUC-NS2A	*Msc*I	CCTTGGCcATTCCAGcaACAATGAC
36	4812	G > A	Val > Ile	NS3	pUC-NS2A	*Apo*I	GACGTTCAaaTttTaGCCATAGAACC
37	4891	U > C	Ile > Thr	NS3	pUC-NS2A	*Kas*I	CTGGAGAAAcgGGcGCcGTAACATTAG
38	4896	G > U	Ala > Ser	NS3	pUC-NS2A	*Bst*EII	GAAATTGGAtCgGTAACcTTAGATTTC
39	4907	A > U	Leu > Phe	NS3	pUC-NS2A	*Acl*I	GGAGCAGTAACgTTtGATTTCAAACCC
40	4995	U > C	Ser > Pro	NS3	pUC-NS2A	*Bsa*JI	GTTACCAAAcCtGGgGATTACGTC
41	5097	G > A	Asp > Asn	NS3	pUC-NS3	*Bsp*HI	GATTAACTATcATGaACTTACACCC
42	5695	A > G	Asp > Gly	NS3	pUC-NS3	*Ban*I	GGAAAACCTTTGgcACcGAGTATCC
43	5872	C > U	Thr > Ile	NS3	pUC-NS3	*Bsr*FI	TCCAGTGAtaCCgGCtAGCGCTGCTC
44	6106	A > G	Glu > Gly	NS3	pUC-NS3	*Msc*I	GCCTCAGAGGtGgcCAAAGGAAG
45	6259	U > C	Val > Ala	NS3	pUC-NS3	*Bgl*II	ACATGGAGGcaGAgATcTGGACTAGA
46	7153	U > C	Val > Ala	NS4B	pUC-NS4A	*Msc*I	AAAGCATGgCcAAGGATGCTGTC
47	7162	U > C	Leu > Ser	NS4B	pUC-NS4A	*Blp*I	GCATAATGGACgctAAGCATGACTAAGG
48	7163	A > C	Leu > Phe	NS4B	pUC-NS4A	*Apa*LI	TTATTGCATAgTGcACgAAAAGCATG

SEQ ID NO.	Recombinant virus (rDEN4-)	Nucleotide change	Amino acid change	Gene	pUC clone	RE site[a]	Oligonucleotide[b]
49	7174	C > U	Ala > Val	NS4B	pUC-NS4A	*Bsa*AI	GGGCCTATTATTaCgTAATGGAC
50	7182	G > A	Gly > Ser	NS4B	pUC-NS4A	n/a	CTGCAATCCTGGtgaTATTATTGC
51	7546	C > U	Ala > Val	NS4B	pUC-NS5A	*Acl*I	CTCATAAAGAAcGttCAAACCCT
52	7630	A > G	Lys > Arg	NS5	pUC-NS5A	*Hga*I	CATTAGACAGAcgcGAGTTTGAAG
53	7849	A > U	Asn > Ile	NS5	pUC-NS5A	*Hga*I	TGGCGACgCTCAAGAtaGTGACTGAAG
54	8020	A > U	Asn > Ile	NS5	pUC-NS5A	*Cla*I	GAGTCATCaTCgAtaCCAACAATAG
55	8092	A > G	Glu > Gly	NS5	pUC-NS5A	*Eco*RI	CTTCAAAACCTGgcTTCTGCATCAAAG
56	8281	U > C	Leu > Ser	NS5	pUC-NS5B	*Xmn*I	CAAAGATGTTGagcAACAGGTTCACAAC
57	8730	A > C	Asn > His	NS5	pUC-NS5B	*Ava*I	GGAAAGAAGAAAcAcCCgAGACTGTGC
58	8872	A > G	Lys > Arg	NS5	pUC-NS5B	*Pvu*I	GGGAACTGGTcGAtcgAGAAAGGGC
59	9977	G > A	Met > Ile	NS5	pUC-NS5C	*Sfc*I	CCAGTGGATtACtACaGAAGATATGCTC
60	10186	U > C	Ile > Thr	NS5	pUC-NS5C	*Age*I	CAGGAACCTGAcCGGtAAAGAGGAATACG
61	10275	A > U	n/a	3' UTR	pUC-NS5C	n/a	CTGTAATTACCAACAtCAAACACCAAAG
62	10279	A > C	n/a	3' UTR	pUC-NS5C	n/a	CCAACAACAAcCACCAAAGGCTATTG
63	10634	U > C	n/a	3' UTR	pUC-3'UTR	n/a	GGATTGGTGTTGTcGATCCAACAGG

[a] Primers were engineered which introduced (underline) or ablated (hatched line) translationally-silent restriction enzyme sites.

[b] Lowercase letters indicate nt changes and bold letters indicate the site of the 5-FU mutation, which in some oligonucleotides differs from the original nucleotide substitution change in order to create a unique restriction enzyme site. The change preserves the codon for the amino acid substitution.

TableE 13. *sp, ts* and mouse attenuation phenotypes of rDEN4 mutant viruses encoding single mutations identified in six *sp* 5-FU mutant viruses.

5-FU mutant virus	Virus	Gene/ region containing mutation	Mean virus titer (log_{10}PFU/ml) at indicated temp (°C)						Replication in suckling mice[b]			Replication in HuH-7-SCID mice[d]		
			Vero cells			HuH-7 cells			n	Mean virus titer ± SE (log_{10}PFU /g brain)	Mean log_{10}-unit reduction from value for wt[c]	n	Mean peak virus titer ± SE (log_{10} PFU/ml serum)	Mean log_{10}-unit reduction from value for wt[c]
			35	39	Δ[a]	35	39	Δ						
	2A-13		7.6	7.1	0.5	7.8	6.6	1.2	30	6.5 ± 0.1	—	29	6.8 ± 0.2	—
	rDEN4		7.6	6.8	0.8	8.0	6.7	1.3	54	5.8 ± 0.1	—	32	6.3 ± 0.2	—
	rDEN4Δ30		7.6	6.9	0.7	7.7	5.6	2.1	30	5.6 ± 0.1	0.2	18	5.4 ± 0.2	0.9
738	parent		6.5	5.7	0.8	x6.9	3.1[e]	3.8	12	4.4 ± 0.4	**2.3**	9	5.4 ± 0.7	1.9
	rDEN4-3540	NS2A	6.9	5.1	1.8	7.4	3.7	3.7	12	4.1 ± 0.3	**1.7**	5	6.1 ± 0.3	(+)0.1
	rDEN4-7162	NS4B	7.2	6.8	0.4	7.4	6.6	0.8	8	5.6 ± 0.3	0.3	5	6.8 ± 0.6	0.3
922	parent		7.3	3.8	3.5	x7.4	3.0	4.4	12	3.5 ± 0.1	**2.9**	6	6.2 ± 0.2	0.4
	rDEN4-4306	NS2B	x5.0	2.2	2.8	x5.6	≤1.6	>4.0	12	1.7 ± 0.1	**4.1**	5	5.2 ± 0.6	1.1
	rDEN4-5872	NS3	5.7	2.5	3.2	x6.5	≤1.6	>4.9	12	4.5 ± 0.3	1.3	5	6.2 ± 0.5	0.1
	rDEN4-7163	NS4B	7.8	7.2	0.6	8.0	7.4	0.6	6	6.2 ± 0.2	(+)0.1	6	5.8 ± 0.6	(+)0.2
	rDEN4-10279	3' UTR	6.9	5.7	1.2	7.7	5.7	2.0	6	4.8 ± 0.2	0.7	4	6.7 ± 0.2	0.4
1081	parent		6.9	3.9	3.0	x5.8	1.9	3.9	12	2.6 ± 0.2	**3.9**	4	4.2 ± 0.5	**2.4**
	rDEN4-2650	NS1	5.1	3.0	2.1	x5.5	2.8	2.7	12	3.0 ± 0.3	**2.8**	6	4.7 ± 0.5	**2.2**
	rDEN4-7163	NS4B	7.8	7.2	0.6	8.0	7.4	0.6	6	6.2 ± 0.2	(+)0.1	6	5.8 ± 0.6	(+)0.2
1083	parent		7.4	5.8	1.6	x7.4	≤1.6	≥5.8	12	4.5 ± 0.4	**2.0**	9	4.4 ± 0.3	**2.9**
	rDEN4-3702	NS2A	6.8	5.6	1.2	7.6	4.7	2.9	18	4.9 ± 0.3	0.9	7	6.3 ± 0.3	0.2
	rDEN4-7153	NS4B	7.7	7.2	0.5	8.0	6.9	1.1	6	5.7 ± 0.1	0.2	4	5.9 ± 0.7	0.1
	rDEN4-	3' UTR	4.9	1.6	3.3	x5.7	≤1.6	≥4.1	12	2.4 ± 0.3	**3.4**	7	3.3 ± 0.4	**3.6**

			Mean virus titer (log_{10}PFU/ml) at indicated temp (°C)						Replication in suckling mice[b]			Replication in HuH-7-SCID mice[d]		
			Vero cells			HuH-7 cells								
	10634													
1136	parent		5.1	<u>≤1.6</u>	<u>≥</u>3.5	x5.7	<u>≤1.6</u>	<u>≥</u>4.1	6	2.9 ± 0.3	**4.5**	7	4.5 ± 0.4	1.2
	rDEN4-3771	NS2A	7.0	4.6	2.4	x7.6	<u>3.7</u>	3.9	12	2.6 ± 0.4	**3.2**	4	6.4 ± 0.2	(+)0.1
	rDEN4-4891	NS3	7.1	<u>≤1.6</u>	>5.5	x7.4	<u>≤1.6</u>	>5.8	12	2.5 ± 0.3	**3.5**	6	6.0 ± 0.5	0.3
	rDEN4-10275	3' UTR	6.9	5.8	1.1	7.1	5.2	1.9	6	5.0 ± 0.3	0.5	4	6.7 ± 0.3	0.4
1189	parent		x6.3	<u>3.8</u>	2.5	x5.5	<u>≤1.6</u>	<u>≥</u>3.9	12	3.2 ± 0.4	**3.7**	13	2.3 ± 0.3	**3.8**
	rDEN4-3303	NS1	6.1	4.8	1.3	6.6	3.9	2.7	8	5.7 ± 0.4	0.2	4	6.3 ± 0.3	0.8
	rDEN4-4812	NS3	7.0	6.3	0.7	7.1	6.3	0.8	12	4.8 ± 0.2	1.0	5	6.1 ± 0.5	(+)0.5
	rDEN4-5097	NS3	x5.0	<u>≤1.6</u>	>3.4	x4.6	<u>≤1.6</u>	>3.0	12	1.8 ± 0.1	**4.0**	8	1.9 ± 0.1	**4.3**
	rDEN4-7182	NS4B	7.7	6.9	0.8	7.8	6.8	1.0	6	6.2 ± 0.1	(+)0.1	6	6.3 ± 0.3	(+)0.7

[a] Reduction in mean virus titer (log_{10}PFU/ml) at 39°C compared to permissive temperature (35°C).

[b] Groups of 6 suckling mice were inoculated i.c. with 10^4 PFU of virus. Brains were removed 5 days later, homogenized, and titered in Vero cells.

[c] Comparison of mean virus titers of mice inoculated with mutant virus and concurrent DEN4 control. Bold denotes ≥50- or ≥100-fold decrease in replication in suckling or SCID-HuH-7 mice, respectively.

[d] Groups of HuH-7-SCID mice were inoculated directly into the tumor with 10^4 PFU virus. Serum was collected on day 6 and 7 and titered in Vero cells.

[e] Underlined values indicate a 2.5 or 3.5 log_{10}PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temp when compared to permissive temp (35°C).

[x] Small plaque size at 35°C; small plaques have a diameter of <1.0 mm compared to wild type plaque diameter of 1.5 - 2.0 mm in Vero cells, or a diameter of <0.4 mm compared to wild type plaque diameter of 0.75 to 1.0 mm in HuH-7 cells.

Table 14. Phenotypes of rDEN4 mutant viruses encoding single mutations identified in 10 5-FU mutant viruses that are *ts* in both Vero and HuH-7 cells.

5-FU mutant viruses	rDEN4-Mutation (nt position)	Gene/ region	Mean virus titer (log_{10}PFU/ml) at indicated temp (°C)										Replication in 7-day mice[b]		Replication in HuH-7-SCID mice[d]	
			Vero cells					HuH-7 cells					n	Mean log_{10} reduction from *wt* (log_{10}PFU/g brain)	n	Mean log_{10} reduction from *wt* (log_{10}PFU/ml serum)
			35	37	39	39	Δ[a]	35	37	38	39	Δ				
239, 489	parent		7.6	6.8	5.6	3.3[e]	4.3	7.6	6.7	4.7	2.5	5.1	30	**2.1**	6	0.3
773	4995[f]	NS3	5.7	4.9	3.6	≤1.6	>4.1	6.4	5.7	4.0	≤1.6	>4.8	6	**2.9**		
473	parent		6.7	6.3	5.4	2.0	4.7	7.2	6.7	3.7	1.9	5.3	12	**1.2**	8	(+)0.3
	4480	NS2B	6.7	6.3	6.0	5.7	1.0	7.6	7.2	6.0	5.2	2.4	6	0.7		
	4995[f]	NS3	5.7	4.9	3.6	≤1.6	>4.1	6.4	5.7	4.0	≤1.6	>4.8	6	**2.9**		
759	parent		7.2	6.9	6.4	4.7	2.5	7.5	6.8	6.3	3.1	4.4	12	**1.4**	5	(+)0.4
	4995[f]	NS3	5.7	4.9	3.6	≤1.6	>4.1	6.4	5.7	4.0	≤1.6	>4.8	6	**2.9**		
	8020	NS5	7.1	6.6	6.7	5.9	1.2	7.4	7.1	6.1	5.4	2.0	6	0.5		
816	parent		6.8	6.4	5.8	3.9	2.9	7.5	6.2	5.5	3.1	4.4	6	**2.9**	6	0.4
	4995[f]	NS3	5.7	4.9	3.6	≤1.6	>4.1	6.4	5.7	4.0	≤1.6	>4.8	6	**2.9**		
	7174	NS4B	6.9	7.1	6.9	6.1	0.8	7.5	7.2	7.1	5.6	1.9	6	0.6		
938	parent		7.1	6.5	5.6	3.1	4.0	7.2	6.4	5.6	3.1	4.1	6	**1.7**	6	0.5
	3442	NS1	5.1	3.6	4.3	2.1	3.0	5.9	4.9	3.9	≤1.6	4.3	6	**4.1**		
	4995[f]	NS3	5.7	4.9	3.6	≤1.6	>4.1	6.4	5.7	4.0	≤1.6	>4.8	6	**2.9**		
	10275	3' UTR	6.9	6.4	6.4	5.8	1.1	7.1	6.8	7.1	5.2	1.9	6	0.5		
173	parent		7.0	6.1	3.2	2.9	4.1	7.0	3.2	3.0	2.1	4.9	18	**2.2**	6	1.1
	7163	NS4B	7.8	7.7	7.6	7.2	0.6	8.0	7.7	7.5	7.4	0.6	6	(+)0.1		
	7849	NS5	7.0	6.7	3.7	2.1	4.9	7.7	5.5	3.6	2.4	5.3	6	**3.1**		
	8872	NS5	7.0	6.3	6.4	4.4	2.6	7.4	6.4	5.1	2.9	4.5	6	0.1		
509	parent		6.2	5.8	5.5	3.4	2.8	6.5	6.1	4.5	≤1.6	>4.9	6	**1.9**	6	**1.5**
	4266	NS2B	5.9	6.1	6.1	5.2	0.7	6.7	6.1	5.7	5.3	1.4	6	1.0		

	8092	NS5	5.0[x]	4.6	4.6	<u>≤1.6</u>	>3.4	5.6[x]	4.8	4.4	<u>≤1.6</u>	>4.0	12	**4.0**		
1033	parent		6.7	6.0	5.9	<u>4.1</u>	2.6	6.9	5.6	4.7	<u>≤1.6</u>	>5.3	12	**1.7**	5	0.7
	4907	NS3	6.7	6.0	5.8	<u>4.0</u>	2.7	7.1	6.1	6.8	<u>2.3</u>	4.8	12	**1.8**		
	8730	NS5	7.0	6.7	6.6	6.7	0.3	7.6	7.0	7.2	6.6	1.0	12	0.6		
	9977	NS5	5.6	5.5	4.6	4.1	1.5	6.4	6.1	6.2	4.6	1.8	6	0.7		

[a] Reduction in mean virus titer (log_{10}PFU/ml) at 39°C compared to permissive temperature (35°C).

[b] Groups of 6 suckling mice were inoculated i.c. with 10^4 PFU of virus. Brains were removed 5 days later, homogenized, and titered in Vero cells.

[c] Comparison of mean virus titers of mice inoculated with mutant virus and concurrent DEN4 control. Bold denotes ≥50- or ≥100-fold decrease in replication in suckling or SCID-HuH-7 mice, respectively.

[d] Groups of HuH-7-SCID mice were inoculated directly into the tumor with 10^4 PFU virus. Serum was collected on day 6 and 7 and titered in Vero cells.

[e] Underlined values indicate a 2.5 or 3.5 log_{10}PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temp when compared to permissive temp (35°C).

[f] Data represents the results from a single rDEN4-4995 virus.

[x] Small plaque size at 35°C; small plaques have a diameter of <1.0 mm compared to wild type plaque diameter of 1.5 - 2.0 mm in Vero cells, or a diameter of <0.4 mm compared to wild type plaque diameter of 0.75 to 1.0 mm in HuH-7 cells.

Table 15. *sp, ts* and mouse attenuation phenotypes of rDEN4 mutant viruses encoding single mutations identified in 3 HuH-7 cell-specific *ts* 5-FU mutant viruses.

5-FU mutant viruses	rDEN4-Mutation (nt position)	Gene/ region	Mean virus titer (log_{10}PFU/ml) at indicated temp (°C)										Replication in 7-day mice[b]		Replication in HuH-7-SCID mice[b]	
			Vero cells					HuH-7 cells					n	Mean log_{10} reduction from *wt*[c] (log_{10}PFU/g brain)$_{10}$	n	Mean log_{10} reduction from *wt*[c] (log_{10}PFU/ml serum)
			35	37	39	39	Δ[a]	35	37	38	39	Δ				
686	parent		7.0	6.7	6.7	6.4	0.6	7.3	6.8	6.4	2.2	5.1	12	**3.8**	6	1.2
	3575	NS2A	6.9	6.9	7.1	7.0	0.1	7.9	6.8	6.9	4.9	3.0	12	**2.3**		nd[e]
	4062	NS2A	6.8	6.6	6.3	4.7	2.1	6.9	6.8	7.0	≤1.6	>5.3	12	**2.2**		nd
	7163	NS4B	7.8	7.7	7.6	7.2	0.6	8.0	7.7	7.5	7.4	0.6	6	(+)0.1		nd
992	parent		7.3	7.1	6.8	5.9	1.4	7.4	6.9	5.0	≤1.6	>5.8	6	**2.7**	7	1.3
	5695	NS3	5.6	4.7	4.7	3.8	1.8	6.3	5.1	3.7	≤1.6	>4.7	6	**2.8**		nd
	7162	NS4B	7.2	7.3	6.6	6.8	0.4	7.4	7.3	7.3	6.6	0.8	8	0.3		nd
1175	parent		7.4	7.1	6.9	5.3	2.1	7.6	6.5	4.7	3.3	4.3	12	**1.7**	5	1.0
	7153	NS4B	7.7	7.7	7.6	7.2	0.5	8.0	7.8	7.5	6.9	1.1	6	0.2		nd
	10186	NS5	4.3	3.7	2.4	≤1.6	>2.7	5.1	≤1.6	≤1.6	≤1.6	>3.5	6	**3.4**		nd
	10275	3' UTR	6.9	6.4	6.4	5.8	1.1	7.1	6.8	7.1	5.2	1.9	6	0.5		nd

[a] Reduction in titer (log_{10}PFU/ml) at 39°C compared to permissive temperature (35°C).

[b] Groups of 6 suckling mice were inoculated i.c. with 10^4 PFU virus. Brains were removed 5 days later, homogenized, and titered in Vero cells.

[c] Determined by comparing mean viral titers of mice inoculated with mutant virus and concurrent 2A-13 or rDEN4 wt control.

[d] Underlined values indicate a 2.5 or 3.5 log_{10}PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temp when compared to permissive temp (35°C).

Table 16. Temperature-sensitive (*ts*) and mouse brain attenuation (*att*) phenotypes of additional rDEN4 viruses encoding single 5-FU mutations.

5-FU mutant virus	Virus	Gene/ region containing mutation	Mean virus titer (log_{10}PFU/ml) at indicated temp (°C)										n	Replication in suckling mice[b]	
			Vero cells					HuH-7 cells						Mean virus titer ±SE (log_{10}PFU/g brain)	Mean log_{10} -unit reduction from value for wt[c]
			35	37	38	39	Δ[a]	35	37	38	39	Δ			
695	rDEN4-40	5' UTR	7.4	7.2	6.7	6.2	1.2	7.6	7.5	7.1	5.8	1.8		nd[f]	nd
718	rDEN4-4059	NS2A	7.0	6.7	6.4	6.2	0.8	7.7	7.1	7.0	6.6	1.1		nd	nd
311	rDEN4-4896	NS3	7.0	6.1	5.9	4.2	2.8	6.9[x]	6.0	5.6	3.3	3.6	6	4.1 ± 0.4	**2.0****
695	rDEN4-6106	NS3	6.8	6.3	5.9	3.9	2.9	7.1	6.0	5.2	3.4	3.7		nd	nd
631	rDEN4-6259	NS3	7.0	6.1	5.8	5.0	2.0	7.5	6.6	5.7	4.2	3.3	6	2.2 ± 0.2	**3.9****
695[e]	rDEN4-7546	NS4B	7.5	7.6	7.4	6.6	0.9	7.7	7.6	7.3	5.7	2.0		nd	nd
718	rDEN4-7630	NS5	7.0	6.9	6.9	6.4	0.6	7.4	7.4	7.2	6.8	0.6	6	5.0 ± 0.3	0.5
718	rDEN4-8281	NS5	6.4	6.6	6.7	5.4	1.0	7.6	7.6	7.0	5.1	2.5	6	5.0 ± 0.5	1.1

[a] Reduction in titer (log_{10}PFU/ml) at 39°C compared to titer at permissive temperature (35°C).

[b] 6 mice were inoculated i.c. with 10^4 PFU virus in 30µl inoculum. Brains were removed 5 days later, homogenized, and titered on Vero cells. Limit of detection is 2.0 log_{10}PFU/g.

[c] Determined by comparing mean viral titers of mice inoculated with sample virus and wt rDEN4 control.

[d] Underlined values indicate a 2.5 or 3.5 log_{10}PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temperature when compared to permissive temperature (35°C).

[e] The 7546 mutation is also present in nine other 5-FU mutant viruses.

[x] Small plaque size at 35°C; small plaques have a diameter of <0.4 mm compared to wt plaque diameter of 0.75 to 1.0 mm in HuH-7 cells.

[f] not determined

** The *att* phenotype is defined as a reduction of >1.5 log_{10}PFU/g compared to wt virus.

Table 17. Growth of wt DEN-4 2A-13 in SCID mice transplanted with HuH-7 cells.[a]

Dose (log_{10}PFU/ml)	Mouse #	Virus titer				
		log_{10}PFU/ml serum		log_{10}PFU/g tissue		
		day 3	day 5	Brain	Liver	Tumor
4	87	2.7	5.9	2.0	6.9	8.0
	88	2.0	5.9	3.8	3.3	8.0
	89	<1.7	6.2	2.7	3.6	8.0
	90	1.7	3.5	3.2	3.0	7.0
5	84	<1.7	7.2	3.2	4.0	7.0
	85	1.7	6.6	3.6	6.3	5.8
6	91	4.4	8.3	6.0	7.3	8.0
	92	4.2	7.7	3.3	6.9	7.3
	93	4.0	6.6	3.3	5.7	8.4
	94	4.3	8.1	5.8	7.8	7.5

[a] SCID mice were injected i.p. with 10^7 HuH-7 human hepatoma cells. Approximately 8 weeks later, groups of tumor-bearing SCID-HuH-7 mice were inoculated with virus directly into the tumor. Serum and tissues were collected on day 5, processed, and titered in Vero cells.

Table 18. Combination of *ts* mutations, NS3 4995 and NS5 7849, in rDEN4 results in an additive *ts* phenotype.

Virus	Mean virus titer (log_{10}PFU/ml) at indicated temp (°C)										Replication in suckling mice[b]	
	Vero cells					HuH-7 cells					Mean virus titer ± SE (log_{10}PFU/g brain)	Mean log_{10} reduction from *wt*[c]
	35	37	38	39	Δ[a]	35	37	38	39	Δ		
2A-13 wt	7.1	7.1	6.9	6.8	0.3	7.4	7.3	6.7	6.4	1.0	6.9 ± 0.09	-
rDEN4 wt	7.0	6.8	6.6	6.4	0.6	7.5	7.3	6.7	6.4	1.1	6.5 ± 0.11	-
rDEN4Δ30	7.0	6.7	6.2	6.2	0.8	7.5	7.0	6.5	5.1	2.4	5.9 ± 0.21	0.6
rDEN4-4995	5.7	4.9	3.6	≤1.6[d]	>4.1	6.4	5.7	4.0	≤1.6	>4.8	3.4 ± 0.10	**3.1**
rDEN4-7849	7.0	6.7	3.7	2.1	4.9	7.7	5.5	3.6	2.4	5.3	2.6 ± 0.29	**3.9**
rDEN4-4995-7849	5.9	2.8	≤1.6	≤1.6	>4.3	5.6	2.4	≤1.6	≤1.6	>4.0	2.3 ± 0.20	**4.2**

[a] Reduction in titer (log_{10}PFU/ml) at 39°C compared to titer at permissive temperature (35°C).

[b] Groups of 6 suckling mice were inoculated i.c. with 10^4 PFU virus. Brains were removed 5 days later, homogenized, and titered in Vero cells. The limit of detection is 2.0 log_{10}PFU/g.

[c] Determined by comparing mean viral titers of mice inoculated with sample virus and rDEN4 wt control.

[d] Underlined values indicate a 2.5 or 3.5 log_{10}PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temperature when compared to permissive temperature.

Table 19. The 5-FU mutations are compatible with the Δ30 mutation for replication in the brain of suckling mice.

Virus	No. of mice/ group	Mean virus titer ± SE (log_{10}PFU/g brain)[a]	Mean log_{10}-unit reduction from wt[b]
rDEN4	12	6.0 ± 0.1	—
rDEN4Δ30	12	5.3 ± 0.1	0.7
rDEN4-2650[c]	12	3.7 ± 0.2	**2.3**
rDEN4Δ30-2650	12	3.9 ± 0.1	**2.1**
rDEN4-4995[d]	6	3.5 ± 0.2	**2.5**
rDEN4Δ30-4995	6	2.7 ± 0.4	**3.3**
rDEN4-8092[d]	12	2.0 ± 0.1	**4.0**
rDEN4Δ30-8092	6	3.2 ± 0.2	**2.8**
rDEN4-10634[c]	12	3.8 ± 0.1	**2.2**
rDEN4Δ30-10634	12	3.6 ± 0.1	**2.4**

[a] Groups of 6 suckling mice were inoculated i.c. with 10^4 PFU of virus. Brains were removed 5 days later, homogenized, and titered in Vero cells.

[b] Comparison of mean virus titers of mice inoculated with mutant virus and rDEN4 control.

[c] Mutation restricts growth in both mouse brain and HuH-7-SCID mice.

[d] Mutation restricts growth in mouse brain only. The 8092 mutation has not been tested in SCID-HuH7 mice.

Table 20. Temperature-sensitive and mouse brain attenuation phenotypes of viruses bearing charge-cluster-to-alanine mutations in the NS5 gene of DEN4.

Mutation[a]	Changed AA Pair	# nt changed	Mean virus titer (log_{10} PFU/ml at indicated temperature (°C)[b]										Replication in suckling mice[d]	
			Vero Cells					HuH-7 Cells					n	Mean titer ± SE (log_{10}PFU/g brain)	Mean log reduction from wt[e]
			35	37	38	39	Δ[c]	35	37	38	39	Δ			
wt (rDEN4)	*n/a*	*0*	*8.1*	*8.1*	*7.9*	*7.6*	*0.5*	*8.3*	*8.0*	*7.5*	*7.5*	*0.8*	48	6.0 ± 0.16	-
deletion (rDEN4Δ30)	*n/a*	*30*	*6.3*	*6.1*	*6.1*	*5.7*	*0.6*	*6.9*	*6.3*	*5.9*	*4.7*	*2.2*	42	5.4 ± 0.22	0.6
21-22	D R	4	7.2	6.8	6.7	6.1	1.1	7.6	7.1	7.0	4.7	2.9	6	5.0 ± 0.50	0.6
22-23	R K	4	7.0	7.8	6.9	3.7	3.3	7.6	7.6	6.5	≤1.7	>5.9	6	2.6 ± 0.19	**2.9**
23-24	K E	3	6.7	6.6	6.0	6.5	0.2	7.1	7.3	5.6	≤1.7	>5.4	18	4.7 ± 0.09	**1.5**
26-27	E E	3	7.8	7.6	6.8	4.0	3.8	8.4	8.2	7.3	4.9	3.5	6	5.7 ± 0.30	+0.1
46-47	K D	3	7.4	7.4	7.3	7.0	0.4	7.8	7.8	7.3	6.8	1.0	6	5.4 ± 0.42	0.5
157-158	E E	3	6.5	7.2	5.1	5.1	1.4	7.6	7.4	5.9	≤1.7	>5.9	6	2.8 ± 0.31	**2.7**
200-201	K H	4	5.3	4.6	5.3	4.1	1.2	5.6	4.9	3.7	≤1.7	>3.9	12	5.5 ± 0.45	0.8
246-247	R H	5	6.9	5.8	5.7	5.4	1.5	6.4	6.1	6.1	5.5	0.9	6	6.1 ± 0.17	+0.5
253-254	E K	4	7.1	6.9	6.8	7.0	0.1	7.9	7.5	7.6	6.8	1.1	6	6.2 ± 0.13	+0.6
356-357	K E	3	7.7	7.6	7.0	7.0	0.7	8.0	7.3	6.4	≤1.7	>6.3	6	3.5 ± 0.58	**2.0**
387-388	K K	5	7.7	6.1	7.0	≤1.7	>6.0	7.0	6.3	7.0	≤1.7	>5.3	6	3.1 ± 0.33	**2.4**
388-389	K K	5	5.1	4.5	≤1.7	≤1.7	>3.4	6.1	5.0	≤1.7	≤1.7	>4.4	6	5.0 ± 0.23	1.4
396-397	R E	4	7.0	7.3	6.5	5.5	1.5	7.5	7.6	7.5	≤1.7	> 5.8	18	5.4 ± 0.35	1.1
397-398	E E	2	7.0	7.1	7.0	3.0	4.0	8.0	7.6	7.0	≤1.7	>6.3	6	6.0 ± 0.22	0.8
436-437	D K	4	4.5	3.3	3.0	2.0	2.5	5.7	4.5	≤1.7	≤1.7	>4.0	12	2.3 ± 0.14	**3.9**
500-501	R E	3	6.6	6.3	5.7	2.3	4.3	7.1	6.5	≤1.7	≤1.7	>5.4	6	6.9 ± 0.49	+0.7
520-521	E E	3	5.6	4.7	4.3	≤1.7	>3.9	6.7	5.7	≤1.7	≤1.7	>5.0	6	5.2 ± 0.48	0.2
523-524	D K	4	6.6	6.3	6.3	5.8	0.8	7.1	6.6	≤1.7	≤1.7	>5.4	6	4.2 ± 0.47	1.3
524-525	K K	5	7.1	6.9	6.9	6.6	0.5	7.8	7.4	7.0	5.3	2.5	6	3.4 ± 0.54	**2.1**
525-526	K D	4	7.8	7.1	7.6	6.8	1.0	7.9	7.7	8.0	6.9	1.0	6	3.7 ± 0.64	**1.8**
596-597	K D	3	4.6	4.0	2.6	≤1.7	>2.9	5.7	4.9	4.0	≤1.7	>4.0	6	5.9 ± 0.14	0.5
641-642	K E	4	7.3	6.9	6.9	5.2	2.1	7.8	7.5	7.2	6.9	0.9	6	4.7 ± 0.45	1.2
642-643	E R	3	6.8	6.1	4.0	3.3	3.5	7.5	7.1	6.6	3.0	4.5	12	2.6 ± 0.15	**3.6**
645-646	E K	4	6.3	5.3	5.9	3.1	3.2	6.4	5.8	5.5	4.5	1.9	6	5.4 ± 0.51	0.2
649-650	K E	3	6.9	6.8	6.9	6.3	0.6	7.1	7.3	7.5	7.0	0.1	12	6.4 ± 0.20	+0.2
654-655	D R	4	6.3	5.7	≤1.7	≤1.7	>4.6	7.0	7.1	4.6	≤1.7	>5.3	12	1.8 ± 0.10	**4.0**

750-751	R E	3	7.1	7.1	6.9	5.7	1.4	7.8	6.9	6.5	5.6	2.2	6	6.0 ± 0.18	0.7
808-809	E D	3	4.6	4.1	<u>≤1.7</u>	<u>≤1.7</u>	>2.9	5.2	<u>≤1.7</u>	<u>≤1.7</u>	<u>≤1.7</u>	>3.5	6	1.8 ± 0.05	**3.1**
820-821	E D	2	6.3	6.3	5.6	<u>≤1.7</u>	>4.6	6.9	6.0	5.7	<u>≤1.7</u>	>5.2	6	5n5 ± 0.33	1.2
827-828	D K	4	6.9	6.3	6.3	5.9	1.0	7.5	6.9	5.0	<u>≤1.7</u>	>5.8	6	3.6 ± 0.76	**2.3**
877-878	K E	3	7.6	7.3	7.0	7.0	0.6	7.9	7.9	7.3	5.8	2.1	12	4.4 ± 0.65	**1.8**
878-879	E E	3	7.6	7.3	7.3	7.1	0.5	8.1	8.1	7.9	6.6	1.5	12	2.4 ± 0.10	**3.8**

[a] Positions of the amino acid pair mutated to an alanine pair; numbering starts at the amino terminus of the NS5 protein.

[b] Underlined values indicate a 2.5 or 3.5 log10 PFU/ml reduction in titer in Vero or HuH-7 cells, respectively, at the indicated temperatures when compared to permissive temperature (35°C).

[c] Reduction in titer (log10 PFU/ml) at 39°C compared to permissive temperature (35°C).

[d] Groups of six mice were inoculated i.c. with 4.0 log10 PFU virus in a 30 µl inoculum. The brain was removed 5 days later, homogenized, and titered in Vero cells.

[e] Determined by comparing mean viral titers in mice inoculated with sample virus and concurrent wt controls (n = 6). The attenuation phenotype is defined as a reduction of ≥1.5 log10 PFU/g compared to wt virus; reductions of ≥1.5 are listed in boldface.

Table 21. SCID-HuH-7 attenuation phenotypes of viruses bearing charge-cluster-to-alanine mutations in the NS5 gene of DEN4.

Mutation[a]	AA changed	Replication in SCID-HuH-7 mice[b]		
		n	Mean peak virus titer ± SE (log_{10}PFU/ml serum)	Mean log reduction from wt[c]
wt	*na*	21	5.4 ± 0.4	-
Δ30	*na*	4	3.7 ± 0.6	**2.5**
23-24	KE	19	4.7 ± 0.5	1.3
157-158	EE	6	4.6 ± 0.6	1.3
200-201	KH	12	3.7 ± 0.2	**2.6**
356-357	KE	10	6.3 ± 0.7	(-) 1.1
396-397	RE	12	4.4 ± 1.3	1.2
397-398	EE	6	6.0 ± 0.5	(-) 0.1
436-437	DK	6	3.6 ± 0.2	**2.6**
500-501	RE	8	5.1 ± 0.4	1.1
523-524	DK	5	5.3 ± 0.7	0.6
750-751	RE	8	5.1 ± 0.4	1.1
808-809	ED	8	3.2 ± 0.4	**3.0**
827-828	DK	5	2.9 ± 0.2	1.6
878-879	EE	5	4.4 ± 0.7	1.5

[a] Positions of the amino acid pair changed to a pair of alanines; numbering starts at the amino terminus of the NS5 protein.

[b] Groups of SCID-HuH-7 mice were inoculated directly into the tumor with 10^4 PFU virus. Serum was collected on days 6 and 7 and titered in Vero cells.

[c] Comparison of mean virus titers of mice inoculated with mutant virus and concurrent DEN4 control. Bold denotes a ≥100-fold decrease in replication. A (-) sign indicates an increase in replication relative to wt.

Table 22. Combination of paired charge-cluster-to-alanine mutations into double-pair mutant viruses.

Mutation Pair 1	Mutation Pair 2	Recovered
23-24	200-201	Yes
23-24	356-357	Yes
23-24	396-397	Yes
23-24	523-524	Yes
23-24	827-828	No
157-158	200-201	No
157-158	356-357	No
157-158	396-397	No
157-158	523-524	Yes
157-158	827-828	No
827-828	200-201	No
827-828	356-357	No
827-828	396-397	Yes
827-828	523-524	No

Table 23. Temperature-sensitive and mouse brain attenuation phenotypes of double charge-cluster-to-alanine mutants of the NS5 gene of rDEN4.

Mutation[a]	Charged AA Pair	#nt changed	Mean virus titer (log10 PFU/ml) at indicated temperature (°C)[b]										Replication in suckling mice[d]		
			Vero Cells					HuH-7 cells					n	Mean virus titer ± SE (log_{10}PFU/g brain)	Mean log reduction from wt[e]
			35	37	38	39	Δ[c]	35	37	38	39	Δ			
wt	*n/a*	*0*	*8.1*	*8.1*	*7.9*	*7.6*	*0.5*	*8.3*	*8.0*	*7.5*	*7.5*	*0.8*	48	6.0 ± 0.16	-
Δ30	*n/a*	*30*	*6.3*	*6.1*	*6.1*	*5.7*	*0.6*	*6.9*	*6.3*	*5.9*	*4.7*	*2.2*	42	5.4 ± 0.22	0.6
23-24	K E	3	6.7	6.6	6.0	6.5	0.2	7.1	7.3	5.6	≤1.7	>5.4	18	4.7 ± 0.09	**1.5**
200-201	K H	4	5.3	4.6	5.3	4.1	1.2	5.6	4.9	3.7	≤1.7	>3.9	12	5.5 ± 0.45	0.8
23-24; 200-201	K E, K H	7	7.1	6.5	6.6	≤1.7	>5.4	7.8	7.3	≤1.7	≤1.7	>6.1	6	5.8 ± 0.16	0.6
23-24	K E	3	6.7	6.6	6.0	6.5	0.2	7.1	7.3	5.6	≤1.7	>5.4	18	4.7 ± 0.09	**1.5**
356-357	K E	3	7.7	7.6	7.0	7.0	0.7	8.0	7.3	6.4	≤1.7	>6.3	6	3.5 ± 0.58	**2.0**
23-24; 356-357	K E, K E	6													
23-24	K E	3	6.7	6.6	6.0	6.5	0.2	7.1	7.3	5.6	≤1.7	>5.4	18	4.7 ± 0.09	**1.5**
396-397	R E	4	7.0	7.3	6.5	5.5	1.5	7.5	7.6	7.5	≤1.7	> 5.8	18	5.4 ± 0.35	1.1
23-24; 396-397	K E, R E	7	6.3	4.9	≤1.7	≤1.7	>4.6	7.1	6.0	5.6	≤1.7	>5.4	6	3.7 ± 0.44	**2.7**
157-158	E E	3	6.5	7.2	5.1	5.1	1.4	7.6	7.4	5.9	≤1.7	>5.9	6	2.8 ± 0.31	**2.7**
396-397	R E	4	7.0	7.3	6.5	5.5	1.5	7.5	7.6	7.5	≤1.7	> 5.8	18	5.4 ± 0.35	1.1
157-158; 396-397	E E, R E	7											6	2.0 ± 0.12	**4.8**
157-158	E E	3	6.5	7.2	5.1	5.1	1.4	7.6	7.4	5.9	≤1.7	>5.9	6	2.8 ± 0.31	**2.7**
523-524	D K	4	6.6	6.3	6.3	5.8	0.8	7.1	6.6	≤1.7	≤1.7	>5.4	6	4.2 ± 0.47	1.3
157-158; 523-524	E E, D K	7	5.6	3.9	≤1.7	≤1.7	> 3.9	6.3	4.1	≤1.7	≤1.7	> 4.6			
396-397	R E	4	7.0	7.3	6.5	5.5	1.5	7.5	7.6	7.5	≤1.7	> 5.8	6	4.8 ± 0.54	**1.6**

827-828	D K	4	6.9	6.3	6.3	5.9	1.0	7.5	6.9	5.0	<u>≤1.7</u>	>5.8	6	3.6 ± 0.76	**2.3**
396-397;827-828	R E, D K	8	7.0	6.5	6.0	<u>≤1.7</u>	5.3	> 6.7	5.7	<u>≤1.7</u>	<u>≤1.7</u>	> 5.0	6	4.7 ± 0.10	1.2

[a] Positions of the amino acid pair mutated to an alanine pair; numbering starts at the amino terminus of the NS5 protein.

[b] Underlined values indicate a 2.5 or 3.5 $\log_{10}$ PFU/ml reduction in titer in Vero or HuH-7 cells respectively, at the indicated temperatures when compared to permissive temperature (35°C).

[c] Reduction in titer ($\log_{10}$PFU/ml) at 39°C compared to permissive temperature (35°C).

[d] Groups of six suckling mice were inoculated i.c. with 4.0 $\log_{10}$PFU virus in a 30 µl inoculum. Brains were removed 5 days later, homogenized, and titered in Vero cells.

[e] Determined by comparing mean viral titers in mice inoculated with sample virus and concurrent *wt* controls ($n = 6$); reductions ≥ 1.5 are listed in boldface.

Table 24. SCID-HuH-7 attenuation phenotypes of double charge-cluster-to-alanine mutants of the NS5 gene of rDEN4.

Mutation[a]	Charged AA Pair	Replication in SCID-HuH-7 mice[b]		
		n	Mean peak virus titer ± SE (log_{10}PFU/ml serum)	Mean log reduction from wt[c]
wt	*n/a*	21	5.4 ± 0.4	-
Δ30	*n/a*	4	3.7 ± 0.6	**2.5**
23-24	K E	19	4.7 ± 0.5	1.3
200-201	K H	12	3.7 ± 0.2	**2.6**
23-24; 200-201	K E, K H	13	3.4 ± 0.1	**2.9**
23-24	K E	19	4.7 ± 0.5	1.3
356-357	K E	10	6.3 ± 0.7	(+) 1.1
23-24; 356-357	K E, K E	4	3.6 ± 0.3	**2.3**
23-24	K E	19	4.7 ± 0.5	1.3
396-397	R E	12	4.4 ± 1.3	1.2
23-24; 396-397	K E, R E	10	3.4 ± 0.5	**3.3**
157-158	E E	6	4.6 ± 0.6	1.3
396-397	R E	12	4.4 ± 1.3	1.2
157-158; 396-397	E E, R E	6	2.2 ± 0.2	**3.6**
157-158	E E	6	4.6 ± 0.6	1.3
523-524	D K	5	5.3 ± 0.7	0.6
157-158; 523-524	E E, D K	3	5.1 ± 0.6	0.8
396-397	R E	12	4.4 ± 1.3	1.2
827-828	D K	5	2.9 ± 0.2	1.6
396-397;827-828	R E, D K	4	4.1 ± 0.7	0.4

[a] Positions of the amino acid pair mutated to an alanine pair; numbering starts at the amino terminus of the NS5 protein.

[b] Groups of SCID-HuH-7 mice were inoculated directly into the tumor with 10^4 PFU of virus. Serum was collected on days 6 and 7 and titered in Vero cells.

[c] Comparison of mean virus titers of mice inoculated with mutant virus and concurrent DEN4 control. Bold denotes a ≥100-fold decrease in replication. A (+) sign indicates an increase in replication relative to wt.

Table 25. Phenotypes (temperature sensitivity, plaque size and replication in mouse brain and SCID-HuH-7 mice) of wt DEN4 and viruses containing the Δ30 and 7129 mutations.

Virus ID	Mutation[a]	Mean virus titer (log_{10} PFU/ml) at indicated temperature (°C)							Replication in suckling mouse brain[c]			Replication in SCID-HuH-7 mice[e]		
		VERO			HUH7			C6/36	n	Mean virus titer ± SE (log_{10}PFU/g brain)	Mean log reduction from wt[d]	n	Mean peak virus titer ± SE (log_{10}PFU/ml serum)[f]	Mean log reduction from wt[d]
		35	39	Δ[b]	35	39	Δ	32						
1-TD-1A	*wt*	7.3	6.8	0.5	8	6.8	1.2	8.3	36	6.1 ± 0.21	-	21	5.4 ± 0.4	-
p4Δ30	*Δ30*	6.6	6.5	0.1	7.4	6.4	1.0		42	5.4 ± 0.22	0.6	4	3.7 ± 0.6	2.5
5-1A1	C7129U	6.7	6.5	0.2	7.5	6	1.5	7.6*	6	6.2 ± 0.30	0.0			
rDEN4-7129-1A	C7129U	7.3	7.0	0.3	7.6	6.3	1.3	7.5*	6	7.2 ± 0.12	(-) 0.4	4	5.4 ± 0.8	(-) 0.8
rDEN4Δ30-7129	C7129U + Δ30	7.0						7.1*						

[a] Position and identity of the mutated nucleotides.

[b] Reduction in titer (log_{10} PFU/ml) at 39°C compared to permissive temperature (35°C).

[c] Groups of six suckling mice were inoculated i.c. with 4.0 log_{10} PFU virus in a 30 µl inoculum. The brain was removed 5 days later, homogenized, and titered in Vero cells.

[d] Determined by comparing mean viral titers in mice inoculated with sample virus and concurrent *wt* controls (n = 6). The attenuation phenotype is defined as a ≥50- or ≥ 100-fold decrease in replication in suckling or SCID-HuH-7 mice, respectively. A (-) sign indicates an increase in replication relative to the *wt* control.

[e] Groups of SCID-HuH-7 mice were inoculated directly into the tumor with 10^4 PFU virus. Serum was collected on days 6 and 7 and titered in Vero cells.

* Small plaque size.

Table 26. The 5-fluorouracil 5-1A1 small plaque mutant demonstrates a restriction of midgut infection following oral infection of *Aedes aegytpi* mosquitoes.

Virus tested	Dose ingested (log_{10}PFU) [a]	No. mosquitoes tested	Midgut-only infection [b]	Disseminated infection [c]	Total no. infected [d,e]
wtDEN4	4.5	19	1 (5%)	17 (89%)	18 (95%)
(2A-13)	3.5	26	9 (35%)	7 (27%)	16 (62%)
	2.5	28	1 (4%)	0	1 (4%)
				OID_{50} = 3.9	**OID_{50} = 3.3**
5-1A1	3.5	34	4 (12%)	2 (6%)	6 (18%)
	2.5	9	0	1 (11%)	1 (11%)
	1.5	23	0	0	0
					$OID_{50} \geq 3.9$

[a] Amount of virus ingested, assuming a 2 µl bloodmeal.

[b] Number (percentage) of mosquitoes with detectable dengue virus antigen in midgut tissue, but no detectable dengue virus antigen in head; mosquitoes were assayed 21 days post-feed, and dengue virus antigen was identified by IFA.

[c] Number (percentage) of mosquitoes with detectable dengue virus antigen in both midgut and head tissue.

[d] Total number (percentage) of mosquitoes with detectable dengue virus antigen.

[e] The proportion of total infections caused by wild type DEN4 was significantly higher than the proportion caused by 5-1A1 (logistic regression, N = 426, $P < 0.0001$). There were too few disseminated infection caused by 5-1A1 to permit statistical analysis.

Table 27. The 5-fluorouracil 5-1A1 small plaque mutant demonstrates a restriction of infection following intrathoracic inoculation of *Toxorhynchites splendens* mosquitoes.

Virus tested	Dose ingested (log_{10}PFU) [a]	No. mosquitoes tested	No (%) infected [c]
wtDEN4	4.0	5	5 (100)
(2A-13)	3.0	4	4 (100)
	2.0	4	1 (25)
			MID_{50}= 2.3 log_{10}PFU
5-1A1	3.0	9	0
	2.0	7	1 (14)
	1.0	7	0
			MID_{50} > 3.0 log_{10}PFU

[a] Amount of virus inoculated in a 0.22 µl inoculum.

[b] Number (percentage) of mosquitoes with detectable dengue virus antigen in head tissue; mosquitoes were assayed 14 days post-inoculation, and dengue virus antigen was identified by IFA.

[c] The proportion of infections caused by wild type DEN4 was significantly higher than the proportion caused by 5-1A1 (logistic regression, N = 36, P < 0.01).

Table 28. Mutagenesis primers for the deletion or swap of sequences in DEN4 showing conserved differences from tick-borne flaviviruses.

DEN4 nucleotides[1]	Type of mutation[2]	Mutagenesis Primer[3]	SEQ ID NO
10508-10530	Δ	CTGGTGGAAGCCCAACACAAAAAC	64
10508-10530	swap	CTGGTGGAAGGAAGAGAGAAATTGGCAACTCCCCAACACAAAAAC	65
10535-10544	Δ	AGACCCCCCCCAAGCATATTGAC	66
10535-10544	swap	AGACCCCCCCCAATATTTCCTCCTCCTATAGCATATTGAC	67
10541-10544	Δ	CCCAACACAAAGCATATTGAC	68

[1] Nucleotides numbered 5' to 3', in the opposite direction from Figure 5.3

[2] Δ: deletion of specified DEN4 nucleotides; swap: exhange of specified DEN4 nucleotides with homologous sequence from Langat

[3] no swap mutation was made for nucleotides 10541-10544

Table 29. Virus titer and plaque size of 3' UTR mutant viruses in Vero and C6/36 cells.

Virus	Vero		C6/36	
	Titer (log_{10} PFU/ml)	Plaque size[1]	Titer (log_{10} PFU/ml)	Plaque size
rDEN4Δ10508-10530	8.1	wt	7.5	wt
rDEN4swap10508-10530	5.4	sp	6.6	wt
rDEN4Δ10535-10544	5.8	wt	7.0	sp
rDEN4swap10535-10544	7.0	wt	7.3	wt
rDEN4Δ10541-10544	6.4	wt	>7.0	wt

[1] Plaque size is designated as equivalent to wild type (wt) or ≤50% of wild type (sp) on the designated cell type.

Table 30. Infectivity of wt DEN4 and 3' UTR mutants for *Toxorhynchites splendens* via intrathoracic inoculation.

Virus	Dose (log_{10}PFU)[a]	No. mosquitoes tested	% Infected[b]	MID_{50} (log_{10} PFU)
rDEN4 wt	3.3	6	83	2.3
	2.3	7	57	
	1.3	6	0	
	0.3	6	0	
rDEN4Δ10508-10530	4.4	8	0	
	3.4	9	11	
	2.4	4	0	

[a] Amount of virus inoculated in a 0.22 µl inoculum.

[b] Percentage of mosquitoes with detectable dengue virus antigen in head tissue; mosquitoes were assayed 14 days post-inoculation, and dengue virus antigen was identified by IFA

Table 31. Infectivity of 3' UTR swap mutant viruses for *Aedes aegypti* fed on an infectious bloodmeal.

Virus Tested	Dose ingested (log_{10}PFU)[a]	No. Mosquitoes Tested	Total No. Infected[b, c]	Disseminated Infections[c,d]
rDEN4	3.8	18	11 (61%)	4 (22%)
	2.8	15	5 (33%)	1 (6%)
	1.8	15	0	0
			OID_{50} = 3.4	**OID_{50} = ≥ 4.2**
rDEN4swap 10535-10544	3.8	25	5 (20%)	2 (8%)
	2.8	25	0	0
	1.8	20	0	0
			OID_{50} = ≥ 4.2	

[a] Amount of virus ingested, assuming a 2 µl bloodmeal.

[b] Number (%) of mosquitoes with detectable dengue virus antigen in the midgut tissue; mosquitoes were assayed either 14 d post-feed and dengue virus antigen was identified by IFA.

[c] At a dose of 3.8 log_{10}PFU, rDEN4swap10535-10544 infected significantly fewer mosquitoes at the midgut than wt rDEN4 (Fisher's exact test, N = 43, P< 0.01), although disseminated infections were not significantly different (Fisher's exact test, N = 43, P=0.38).

[d] Number (%) of mosquitoes with detectable dengue virus antigen in the head tissue.

Table 32. Putative Vero cell adaptation mutations derived from the set of 5-FU mutant viruses and other DEN4 viruses passaged in Vero cells.

Nucleotide position	Gene / region (a.a. #)[b]	5-FU mutant viruses			Other DEN viruses passaged in Vero cells		
		Nucleotide change	Amino acid change	No. of viruses with the mutation	Virus	Nucleotide change	Amino acid change
1455	E (452)	G > U	val > phe	5			
2280[1,2,3]	E (727)	U > C	phe > leu	2			
4891[2,3]	NS3 (1597)	U > C	ile > thr	2			
4995[1,2]	NS3 (1599)	U > C	ser > pro	8			
7153	NS4B (2351)	U > C	val > ala	3	2AΔ30	U > C	val > ala
7162	NS4B (2354)	U > C	leu > ser	4	2A-1	U > C	leu > ser
7163	NS4B (2354)	A > U or C	leu > phe	7	rDEN4Δ30	A > U	leu > phe
					2A-13-1A1	A > U	leu > phe
7182[1,2,3]	NS4B (2361)	G > A	gly > ser	2			
7546	NS4B (2482)	C > U	ala > val	10			
7630[3]	NS5 (2510)	A > G	lys > arg	1	814669	A > G	lys > arg
10275	3' UTR	A > U	n/a[c]	6			
10279	3' UTR	A > C	n/a	4			

[a] Conservation with DEN1, DEN2, or DEN3 is designated by superscript. Lack of conservation is designated by no superscript.

[b] Amino acid position in DEN4 polyprotein beginning with the methionine residue of the C protein (nt 102-104) as residue #1.

[c] not applicable

Table 33. Sequence analysis of rDEN2/4Δ30 clone 27(p4)-2-2A2.

Nucleotide	Gene	Mutation	
		Nucleotide	Amino acid
743	M anchor	G > A	Gly > Glu
1493	E	C > U	Ser > Phe
7544*	NS4B	C > U	Ala > Val

* Same as DEN4 nucleotide position 7546

Table 34. Sequence analysis of rDEN2/4Δ30 clone 27(p3)-2-1A1.

Nucleotide	Gene	Mutation	
		Nucleotide	Amino acid
1345	E	U > C	Tyr > His
4885*	NS3	G > A	Glu > Lys
8297	NS5	G > A	Arg > Lys

*Codon adjacent to 5-FU mutation 4891

Table 35. Recombinant virus rDEN2/4Δ30 bearing Vero adaptation mutations can be recovery and titered on Vero cells.

Virus	Virus titer in indicated cell line[1] (log_{10}PFU/ml)		Virus titer following recovery in Vero cells (log_{10}PFU/ml)
	C6/36	Vero	
rDEN2/4Δ30 wt	5.2	1.7	<0.7
rDEN2/4Δ30-7153	5.4	5.2	<0.7
rDEN2/4Δ30-7162	5.4	5.3	nd[2]
rDEN2/4Δ30-7182	4.7	4.9	2.3
rDEN2/4Δ30-7630	5.3	4.8	1.3
rDEN2/4Δ30-7153-7163	5.1	4.7	nd
rDEN2/4Δ30-7153-7182	4.1	3.2	nd
rDEN2/4Δ30-7546-7630	5.2	5.2	nd

[1] Virus recovered following transfection of C6/36 mosquito cells was terminally diluted once in C6/36 cells and titered simultaneously in C6/36 cells and Vero cells.

[2] not determined

Table 36. Putative Vero cell adaptation mutations of dengue type 4 virus and the corresponding wildtype amino acid residue in other dengue viruses.

Mutation	Amino acid position[a]	Mutant residue	Amino acid in indicated wt dengue virus[b]			
			DEN4	DEN1	DEN2	DEN3
1455	452	F	V	I	A	A
2280	727	L	F[c]	F	F	F
4891	1597	T	I	V	I	I
4995	1632	P	S	S	S	N
7129	2343	L	P	P	P	P
7153	2351	A	V	F	F	L
7162	2354	S	L	V	V	V
7163	2354	F	L	V	V	V
7182	2361	S	G	G	G	G
7546	2482	V	A	L	T	V
7630	2510	R	K	S	S	K

[a] Amino acid position is given for the polyprotein of DEN4

[b] DEN4 = rDEN4 (GenBank AF326825); DEN1 = Western pacific (GenBank DVU88535); DEN2 = New Guinea C (GenBank AF038403); DEN3 = H87 (GenBank M93130)

[c] Underlined nucleotides are shared between DEN4 and one or more additional DEN types.

Table 37. Mutations known to attenuate dengue type 4 virus and the corresponding wildtype amino acid residue in other dengue virus.

	Mutation	Amino acid position[a]	Mutant residue	Amino acid in indicated wt dengue virus[b]			
				DEN4	DEN1	DEN2	DEN3
5-FU mutations	2650	850	S	N[d]	N	N	N
	3442	1114	G	E	E	E	E
	3540	1147	K	E	E	E	E
	3575	1158	I	M	L	A	M
	3771	1224	G	R	R	K	R
	4062	1321	A	T	L	A	T
	4306	1402	S	N	E	D	D
	4891	1597	T	I	V	I	I
	4896	1599	S	A	A	A	A
	4907	1602	F	L	L	L	L
	4995	1632	P	S	S	S	N
	5097	1666	N	D	D	D	D
	5695	1865	G	D	D	D	D
	6259	2053	A	V	V	V	V
	7129[c]	2343	L	P	P	P	P
	7849	2583	I	N	K	N	K
	8092	2664	G	E	Q	Q	Q
	10186	3362	T	I	I	I	I
	10634	3' UTR	-	-	-	-	-
Charge-cluster-to-alanine mutations	22, 23	2509, 2510	AA	RK	KS	KS	RK
	23, 24	2510, 2511	AA	KE	SE	SE	KE
	157, 158	2644, 2645	AA	EE	EE	EA	EE
	200, 201	2687, 2688	AA	KH	KH	KY	KH
	356, 357	2843, 2844	AA	KE	KE	KE	KE
	387, 388	2874, 2875	AA	KK	RN	KK	RN
	436, 437	2923, 2924	AA	DK	HR	DK	DK
	524, 525	3011, 3012	AA	KK	KI	KK	KI
	525, 526	3012, 3013	AA	KD	IP	KE	IP
	642, 643	3129, 3130	AA	ER	ER	IA	KK
	654, 655	3141, 3142	AA	DR	ER	ER	ER
	808, 809	3295, 3296	AA	ED	ED	ED	ED
	827, 828	3314, 3315	AA	DK	DK	DK	DK
	877, 878	3364, 3365	AA	KE	NE	NE	NE
	878, 879	3365, 3366	AA	EE	EN	EE	EE

[a] Amino acid position is given for the polyprotein of DEN4

[b] DEN4 = rDEN4 (GenBank AF326825); DEN1 = Western pacific (GenBank U88535); DEN2 = New Guinea C (GenBank AF038403); DEN3 = H87 (GenBank M93130)

[c] This mutation results in decreased replication of DEN4 in mosquitoes.

[d] Underlined nucleotides are shared between DEN4 and one or more additional DEN types.